VOLUME XXXIII MARCH, 1954 NUMBER 3

The Journal of Clinical Investigation

EDITED FOR THE AMERICAN SOCIETY FOR CLINICAL INVESTIGATION

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The Journal of Clinical Investigation

Printed by the Lancaster Press, Inc., Prince and Lemon Sts., Lancaster, Pa. Published by the American Society for Clinical Investigation. Publication Office, 622 West 168th St., New York 32, N. Y.

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INTRODUCTION

Excision of lymph nodes is generally recognized as one of the most important considerations in the surgical treatment of patients with visceral carcinoma. Complete removal of these nodes from some regions, however, is often difficult and sometimes impossible. Recognition is made difficult by small size, remote position, or similarity in color and consistency of lymphatic tissues to surrounding areolar or adipose tissues.

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INDICATIONS

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REFERENCES

1. Weinberg, J., and Greaney, E. M.: Identification of regional lymph nodes by means of a vital staining dye during surgery of gastric cancer. Surg., Gynec. & Obst. **90**:561-567 (May) 1950.
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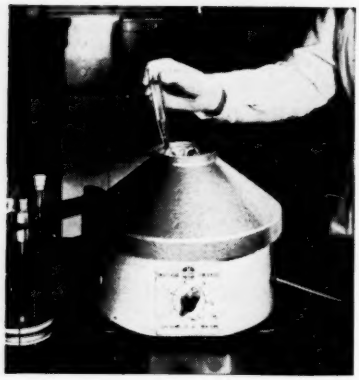

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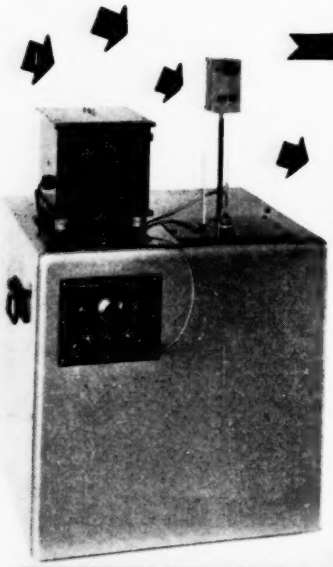

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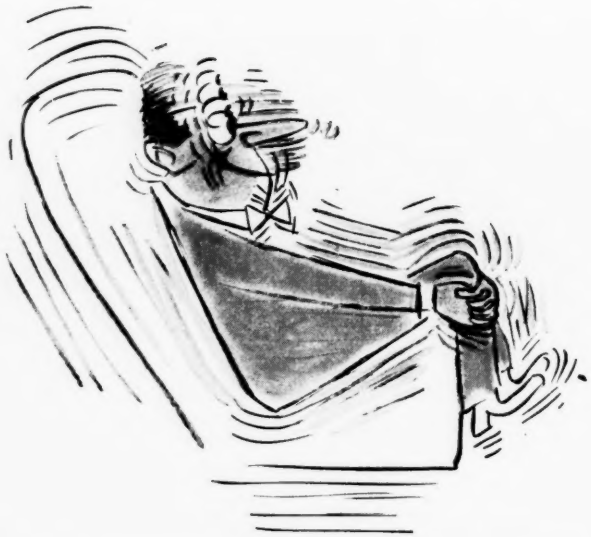

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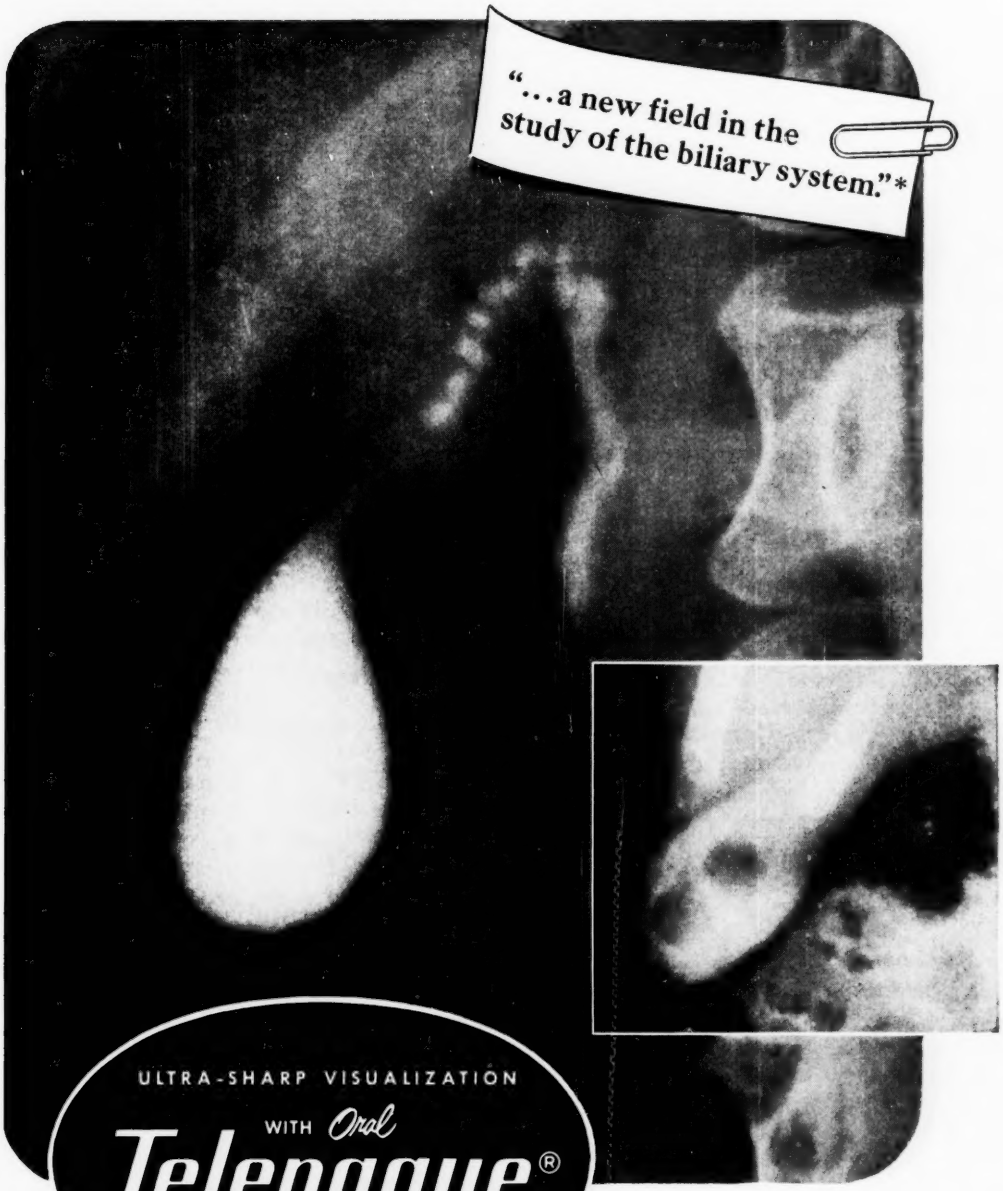

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COLLOID OSMOTIC PRESSURES OF SERUM PROTEINS IN NEPHROSIS AND CIRRHOSIS: RELATIONS TO ELECTROPHORETIC DISTRIBUTIONS AND AVERAGE MOLECULAR WEIGHTS

By S. H. ARMSTRONG, Jr.,[1] R. M. KARK, J. A. SCHOENBERGER, J. SHATKIN, and R. SIGHTS

(From the Department of Medicine, University of Illinois College of Medicine, the Presbyterian Hospital of Chicago, and the Hektoen Institute for Medical Research of the Cook County Hospital of Chicago)

(Submitted for publication July 2, 1953; accepted October 28, 1953)

INTRODUCTION

The albumin component is, by reason of size and net charge and concentration, chiefly responsible for plasmas osmotic activity (1).[2]

The validity of Starling's hypothesis in the dynamics of water balance in conditions associated with hypoproteinemia has been the subject of considerable recent discussion. The basic experimental problems involved have been considered by some of us in other publications (2, 3) and by many other authors (4–11).

The present senior authors, at first separately and now in collaboration, have had the problem of the relation of edema to serum colloid osmotic pressure under experimental observation in a wide variety of clinical situations. We have constantly faced the question: Do we have to measure colloid osmotic pressures, or can we predict from total protein and protein distribution data (usually readily available on metabolic ward patients) with sufficient accuracy to suit our thinking? For although many recent workers have made direct measurements, a certain amount of thinking has been based on colloid osmotic pressures so predicted.

That plasma albumin levels in hypoproteinemic states, whether done by the Howe method (12) or, as has been the case in our more recent studies, by the electrophoretic method (13), can be correlated with osmotic pressures with sufficient precision to elucidate the dynamics of water balance between plasma and interstitial fluid is by no means certain from the recent literature. For example, the conclusion of Keys, Taylor, Mickelsen, and Henschel (14) that the osmotic pressure falls within the normal range in experimentally produced nutritional edema, an opinion based in part on electrophoretic measurements, is at variance with the low osmotic pressures measured directly by Govaerts (15) in war nutritional edemas.

Of the many studies in the last fifteen years (*e.g.*, [16–22]), two may be taken to illustrate representative and sound approaches to the problem in presenting empirical linear equations relating measured osmotic pressures to albumin levels determined by the Howe method over a wide range of values on individual human sera. That of Wells, Youmans, and Miller (21) yields an average variation calculated from observed values of ± 11.56 mm. of water. (The average variation of duplicate osmotic pressure determinations was only ± 2.55 mm. of water.) Again, the best linear fit from the data of Wies and Peters (22) shows an average deviation of ± 27.6 between observed and calculated values. These authors conclude: "Unless practicable methods for the measurement of the colloid osmotic pressure of serum can be greatly improved in accuracy, estimations . . . may be quite as reliable . . . as direct measurements."

[1] This study was initiated during tenure of a Welch Fellowship in Internal Medicine of the National Research Council. Gratitude is expressed to Professor George Scatchard (Department of Chemistry, Massachusetts Institute of Technology) and to Miss J. Weeks of his staff, and to Professor E. J. Cohn (Department of Physical Chemistry, Harvard Medical School) and to Mr. M. J. E. Budka and Miss M. Hasson of his staff, for facilities and help, and to Dr. Murray Franklin of Cook County Hospital for clinical collaboration.

We are indebted to the Otho S. A. Sprague Institute of Chicago for support. The study presently has received grants from the United States Public Health Service.

[2] In this paper osmotic pressure refers exclusively to colloid osmotic pressure or oncotic pressure.

Now, in point of fact, the reproducibility of osmotic pressure measurements obtainable by the use of the Hepp osmometer (23) as modified by Brown, Gee, and others, under Scatchard's direction, in the laboratory of Physical Chemistry at the Massachusetts Institute of Technology (24) is as good as, if not better than, the reproducibility reported by Wells, Youmans, and Miller. Moreover, comparative freedom from systematic errors (when schedule of work permits satisfactorily long equilibration times) has been shown by successful use in the determination of average molecular weights of purified plasma fractions (25).

The question then arises as to whether the difficulty in achieving close correlation with osmotic pressures does not arise from the nature of albumin and globulin measurements on sera. Thus, whereas in our hands the conventional Howe method yields results quite reproducible in the moderate hypoalbuminemia encountered in Laennec's cirrhosis, and good correlation with electrophoretic results has been achieved (26), in the extreme hypoalbuminemias of the nephrotic state we have not been able to attain either this degree of reproducibility or as clear a correlation with the more reproducible electrophoretic measurements (12).

Scatchard, Batchelder, and Brown (27) have proposed a relation between osmotic pressure, total protein concentration, and uncorrected electrophoretic distributions of albumin and globulin, based on measurements on normal pooled human plasma.

We have been interested in whether the reproducibility of electrophoresis (given the arbitrary nature of its analysis) together with Scatchard's relation would yield predictability over a wide range of osmotic pressures and protein distributions associated with certain disease states. In the low pressure range we have studied ascitic fluids of Laennec's cirrhosis in addition to serial sera of three patients with the nephrotic syndrome undergoing spontaneous diuresis induced by measles (28). In the moderately depressed to normal pressure range, we have studied serial sera of seven patients with Laennec's cirrhosis, in addition to sera of normal controls and serial sera on one patient undergoing measles in the absence of nephrosis.

We have also been interested in whether the form of plot $\frac{\text{colloid osmotic pressure}}{\text{protein concentration}}$ *vs.* protein concentration (p/c *vs.* c) might throw any light on the difficulties in correlating measured pressure, protein distributions, and clinical phenomena. We have carried out a number of dilution studies of this type on sera of patients with Laennec's cirrhosis, in the nephrotic phase of adult and childhood chronic glomerulonephritis, and of normal adults.

The electrophoretic distributions in the sera of normal and cirrhotic patients are uncorrected. We have simultaneously measured electrophoretic distributions and specific refractive increments in the sera of patients in the nephrotic state; the latter measurements were made because our previous studies on sera of patients in the nephrotic phase have shown that it is necessary to correct the electrophoretic data for the differences in refractive properties of the albumin and of the lipoproteins which comprise most of the remainder of the electrophoretic diagram. The theoretical basis of these

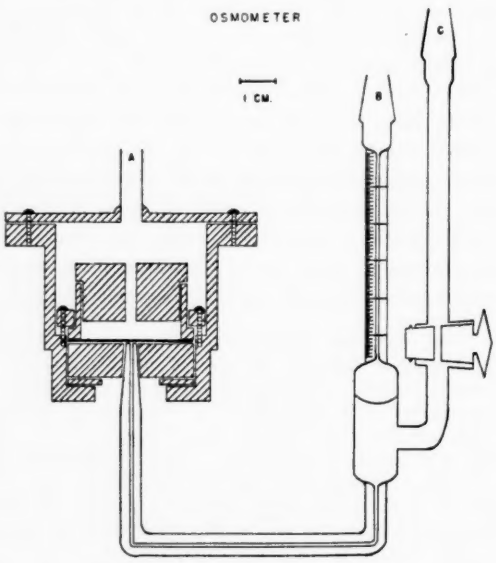


FIGURE 1

An operating description of this instrument, together with the basis for calculations outlined in Figure 2, has been made available in mimeographed form from the Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Mass. (24).

corrections has been presented in previous papers (29, 30).

METHODS

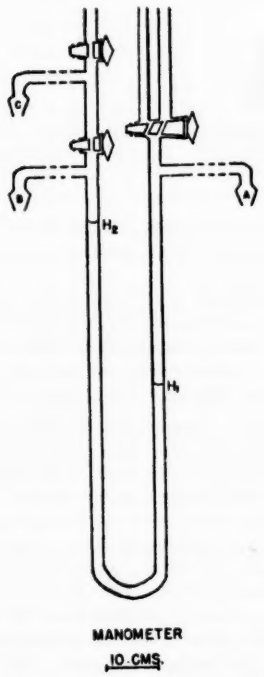
Serum samples, usually taken in the fasting state, were stored frozen at −10° C. until ready for analysis or studied at once. (Osmotic pressures of some individual sera, before and after freezing, were identical within the limits of the method.)

Osmotic pressures were measured directly in the modified Hepp osmometer in equilibrium with .15 M sodium chloride, in a thermostatically controlled bath at 25° C. Membranes of appropriate permeability were prepared of nitrocellulose, according to the method of Pierce (31).

The most satisfactory membranes for our purposes have had a 4 to 1 ethylene glycol nitrocellulose ratio. Of any batch of membranes, a minority will allow enough albumin to leak to warrant discard; the remainder, if kept between uses in physiological saline, have been useful for periods up to six months. Incidence of serious protein leakage is diminished, but equilibration times are increased, with membranes of 3.5 or 3 to 1 ethylene glycol nitrocellulose ratios.

For 4 to 1 membranes, 8.3 Gm. *anhydrous* nitrocellulose (heat dried at 110° and desiccator stored) is weighed quickly into a dry wide mouth ground glass stoppered bottle, and is thoroughly wetted with 32.2 Gm. *anhydrous* ethylene glycol. To this a mixture of 150 ml. *absolute* ethyl alcohol and 50 ml. *anhydrous* ether is added. The stoppered mixture is allowed to stand with periodic shaking until this no longer gives visible refractive index gradients. (A *minimum* of 24 hours is required.) Flat glass plates 3 inches square are floated on mercury in a closed box through which flows a stream of air which has previously passed through both a concentrated H_2SO_4 column and a $CaCl_2$ column. After an hour of dry air passage, 5 ml. of the mixture is pipetted onto each plate and allowed to dry for 48 hours in the dry air current. The resultant membranes separate readily from the plates on dipping in physiological saline; they are thoroughly washed therein to remove all traces of ethylene glycol; cut by scissors to fit the osmometers, and tested for mechanical leaks by hydrostatic pressure; for excessive permeability by determination of protein content of underlying fluid after a 24-hour equilibration against a 10 per cent solution of normal human albumin in the osmometer.

Diagrams of the osmometer are given in Figures 1 and 2. While we often have used the dynamic method of measuring equilibrium (32) (*vide* Figure 2), recently we have preferred to take the additional time required for static equilibrium, particularly for p/c *vs.* c plots, where maximum accuracy at low pressures is essential. Thus



See Figure 1.

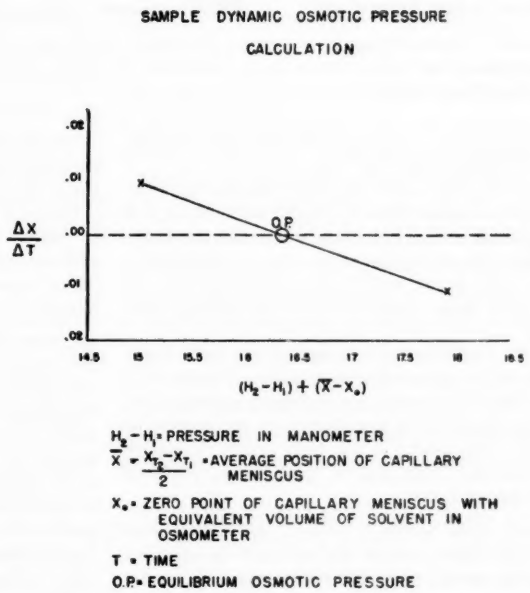


FIGURE 2

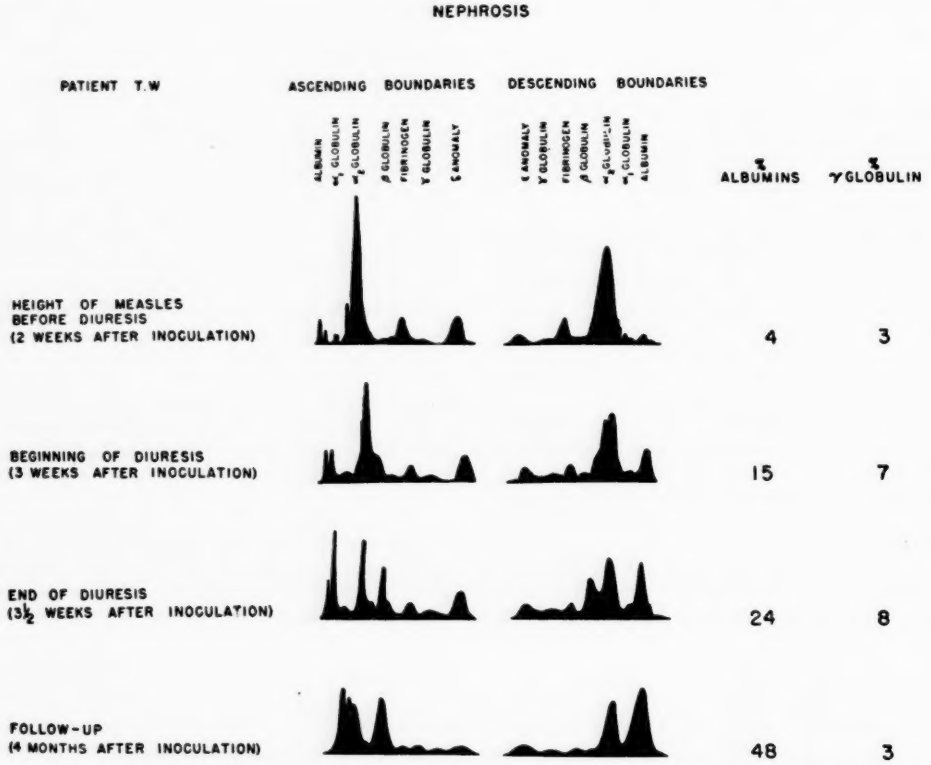


FIG. 3. ELECTROPHORETIC SCHLIEREN DIAGRAMS, TOGETHER WITH PROPORTIONS OF ALBUMINS AND γ-GLOBULINS, OF SERIAL SERA OF A NEPHROTIC PATIENT WITH SPONTANEOUS DIURESIS FOLLOWING MEASLES INOCULATION

many of our pressure measurements have been checked over periods greater than four hours.

For p/c *vs.* c plots sera [3] have been dialyzed for over 24 hours at 5° C. against either 0.15 or 0.3 molal NaCl, and osmotic pressures of serial dilutions, made with the dialysate, have been measured against it. (At 0.3 molal NaCl, the Donnan effect is too small to be of significance in our work; at 0.15 molal NaCl, it is larger though still negligible.)

For p/c *vs.* c plots protein concentrations were measured by the dry weight technique previously described by one of us (30). Otherwise, nondialyzable protein nitrogen was determined by a modification of the method of Pregl (33). The weight of the total protein, exclusive of bound lipid, was estimated by using the conventional nitrogen factor of 6.25.[4]

Electrophoretic analyses were carried out in sodium diethylbarbiturate buffer, pH 8.6, ionic strength of 0.1, in the Tiselius apparatus.

Where refractive indices were measured for correction of electrophoretic distributions, serum aliquots of approximately 8 cc. (whose volumes were known to within .05 cc.) were placed in tight cellophane sacks and dialyzed against 0.3 M sodium chloride. The contents of the sack

[3] Sera with visible lipemia from nephrotic patients were centrifuged at 18,000 r.p.m. in the cold, and the solutions underlying the surface fatty layer were taken for study.

[4] The contribution of this protein to the refractive increment of the protein-lipid combination was estimated by use of a value of 1.17×10^{-4} (in terms of grams of nitrogen per liter of solution). These values for nitrogen factor and specific refractive increment have been found in general to be characteristic of such proteins as albumin and γ-globulin, whose lipid content in the natural state is small. The remainder of the refractive increment has been assumed to be due to protein-bound lipid, and the amount of the latter, in grams per 100 cc., can be estimated by use of a specific increment of 1.40×10^{-4}, in terms of grams per liter of solution, obtained by application of the Lorentz-Lorenz equation (34), in form for binary mixtures, to the data of Dorinson, McCorkle, and Ralston (35). These estimates given in Table I, of course, ignore the possible contribution of bound carbohydrate to the refractive increment of the protein complex.

were then removed, the sack thoroughly washed with the salt solution of composition identified with that against which dialysis was carried out, and the washings added to the contents. With the washings added, measurement of the final volume was carried out to within .05 cc. Original serum concentrations were calculated by application of volume ratios to determinations on dialyzed protein.

Refractive indices of the sample and of the dialysate were measured and specific refractive increments ($\Delta N/\Delta N$ (Gm. prot. nitrogen/liter) $\times 10^5$) were calculated and applied to electrophoretic distributions as previously described (30).

RESULTS

The relation of serum colloid osmotic pressure to refractometrically corrected electrophoretic protein distribution during spontaneous remissions of severe nephrosis

In Figure 3 are presented the electrophoretic Schlieren diagrams of plasmas before, at the beginning of, at the end of, and four months after a typical measles induced diuresis of a nephrotic child. (These plasma diagrams show the fibrinogen component, which is missing from the sera reported in the tables.)

The initial patterns are characterized by very low albumins and gamma globulins, with corresponding elevations of the lipid-rich alpha and beta components. The progression of changes during diuresis closely follows the pattern observed by us (26) in spontaneous diuresis of the nephrotic stage of adult chronic glomerulonephritis, namely a progressive rise both in albumin and gamma globulin, with a diminution in the alpha-beta components and in fibrinogen (which contributes little, by reason of its high molecular weight, to the colloid osmotic pressure). The follow-up diagram four months after inoculation shows a further progression toward an albumin component normal in relative amount, but rather broad in distribution of mobilities, and a persistent reduction of gamma globulins.

In Table I are presented the detailed data on the sera of three patients clinically undergoing the same sequence of events.

A comparison of the uncorrected with the corrected albumin levels shows that the latter are considerably greater than the former.

The relation given by Scatchard and his coworkers is as follows:

$$\frac{p}{c} = \frac{268(1 - .68\,g)}{1 - (.4 + .9\,pH)c}$$

where p is osmotic pressure in mm. Hg $\left(\text{or } \frac{\text{mm. } H_2O}{13.6}\right)$,

c is total protein concentration in Gm./cc.,

g is the ratio $\frac{\text{globulin}}{\text{total protein}}$, and

pH for determinations under routine conditions is taken as 7.4.

(This relation has been corrected by Scatchard [36] for a typographical error in the original publication [27].)

In Table II are given osmotic pressures as measured directly and as calculated by this equation.

The calculated values by no means fall with uniformity within ± 5 mm. H_2O of the experimental values (a range within which, with careful work, our experimental values are reproducible). At the end of diuresis associated with the nephrotic state, the calculated values are notably too low. If electrophoretic distributions uncorrected for refractive increments are used, deviations are even larger.

By simple algebraic transposition of Scatchard's relation, the expression $\frac{p}{c}\left(\frac{1 - (.4 + .9\,pH)c}{268}\right)$ is linear in g; the intercept being unity and the slope being the proportionality coefficient (taken from measurements on normal plasmas and albumin as $-.68$). This linearity implies the assumption of uniform average molecular weights and equivalent effects on pressure of protein-protein interaction for both albumins and globulins. In Figure 4 the values of this expression (based on the direct measurements of pressure in Table II) are plotted against g (based on corrected electrophoretic distributions). Although the points show considerable scatter, for $g > .8$ the available data are also consistent with a slope less, algebraically, than -0.68; moreover, most of the experimental points lie above the predicted line.

The relation of serum colloid osmotic pressure to uncorrected electrophoretic protein distribution of serum and ascitic fluid in hepatic cirrhosis

In Figure 5 are plotted (in fashion identical with Figure 4) experimental points for 27 sera

TABLE I

Protein nitrogens, specific refractive increments, estimated protein-bound lipids, and electrophoretic albumin determinations: nephrosis + measles and measles control (sera)

Patient	Date	Protein nitrogen (Gm./100 cc.)	Protein (Gm./100 cc.)	Δn / ΔN (Gm./liter) ×(10)³	Protein-bound lipid (Gm./100 cc.)	Electrophoretic albumin determinations: Uncorrected %	Uncorrected (Gm./100 cc.)	Corrected for refractive increments %	Corrected for refractive increments (Gm./100 cc.)
F. C.	Before diuresis	0.532	3.33	1.82	2.4	7	0.23	11	0.36
	During diuresis	0.564	3.54	1.79	2.4	10	0.35	15	0.54
	End of diuresis	0.647	4.04	1.70	2.4	12	0.48	17	0.70
A. G.	Before diuresis	0.441	2.76	1.63	1.4	7	0.19	10	0.27
	Before diuresis	0.514	3.21	1.83	2.8	8	0.26	13	0.41
	During diuresis	0.563	3.52	1.78	2.4	11	0.39	17	0.58
	During diuresis	0.821	5.13	1.73	3.3	15	0.77	22	1.14
	End of diuresis	0.856	5.35	1.55	2.3	25	1.34	33	1.77
W. L.	Before diuresis	0.521	3.26	1.65	1.7	6	0.20	8	0.28
	During diuresis	0.645	4.07	1.89	3.3	7	0.29	11	0.46
	End of diuresis	0.858	5.38	1.57	2.4	18	0.97	24	1.30
J. C. (control)	Approximately same periods after measles	1.010	6.32	1.27	0.7	56	3.54	61	3.85
		1.090	6.82	1.26	0.7	53	3.61	57	3.90
		1.070	6.68	1.35	1.3	55	3.68	63	4.25
		1.080	6.76	1.30	1.0	54	3.65	60	4.06

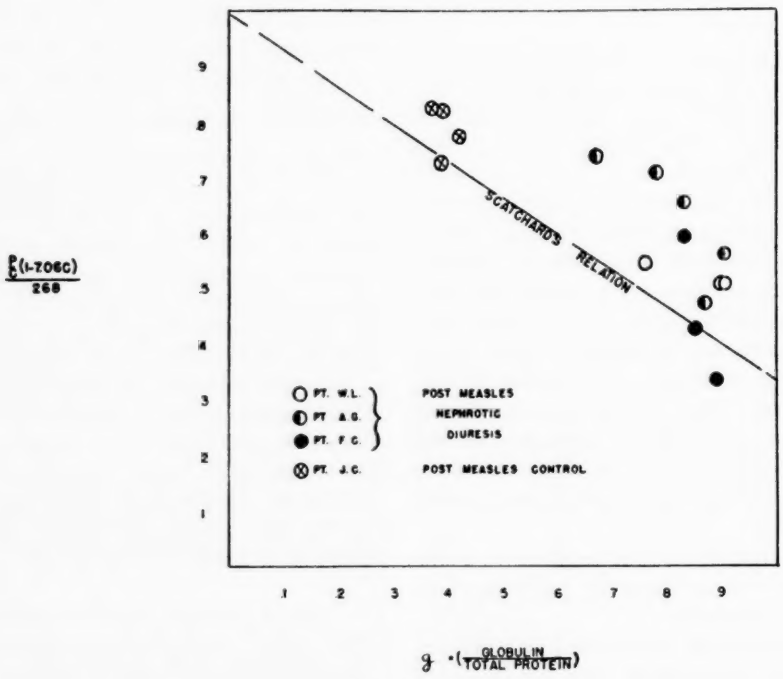


FIGURE 4

For description of this plot see the text.

TABLE II

Osmotic pressures

Patients	Date	Osmotic pressure (*mm. H_2O*) Calculated	Measured
F. C. (nephrosis; post-measles)	Before diuresis	62	50
	During diuresis	72	74
	End of diuresis	90	118
A. G. (nephrosis; post-measles)	Before diuresis	50	71
	Before diuresis	61	72
	During diuresis	81	112
	During diuresis	138	208
	End of diuresis	172	230
W. L. (nephrosis; post-measles)	Before diuresis	58	78
	During diuresis	82	103
	End of diuresis	153	172
J. C. (normal post-measles control)	Approximately same periods after measles	306	339
		342	371
		348	381
		340	334

and 19 ascitic fluids of patients with Laennec's cirrhosis together with 2 sera of normal adult patients. Although the latter lie close to the line of Scatchard's relation, both the sera and the ascitic fluids from cirrhotic patients show wide scatter above and below this. Thus, the relation seems inadequate for prediction of osmotic pressures in Laennec's cirrhosis.

In Figure 6 the osmotic pressures of the same sera of Figure 5 are plotted against the albumin concentrations of these sera in the fashion used by many to calculate regression equations by the method of least squares. The data show so much scatter that such a calculation seems of little value. The scatter is not improved by introducing more complicated functions of the concentrations of the various electrophoretic components (instead of the albumin concentration) as ordinate.

If, however, on Figure 6 a line is drawn from the origin through the two normal values furthermost on the extreme right, one is struck by the fact that the points for most osmotic pressures over 230 mm. of water for sera of patients with Laennec's cirrhosis lie below this line, while for those pressures between 100 and 230 most of the points lie above the line. Indeed, if one eliminates from consideration all the ascitic fluid points, it would appear that in our cirrhotic patients the same range of albumin levels are found associated with quite low osmotic pressures as with pressures in the high range.

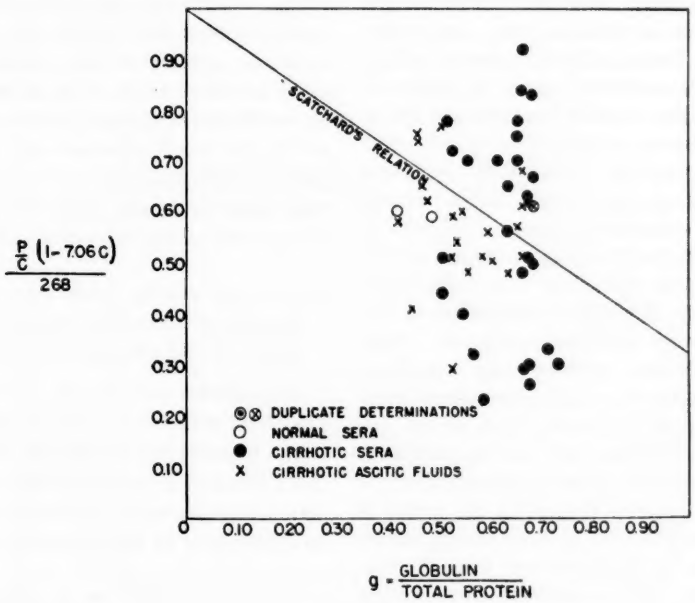


FIGURE 5

For description see text.

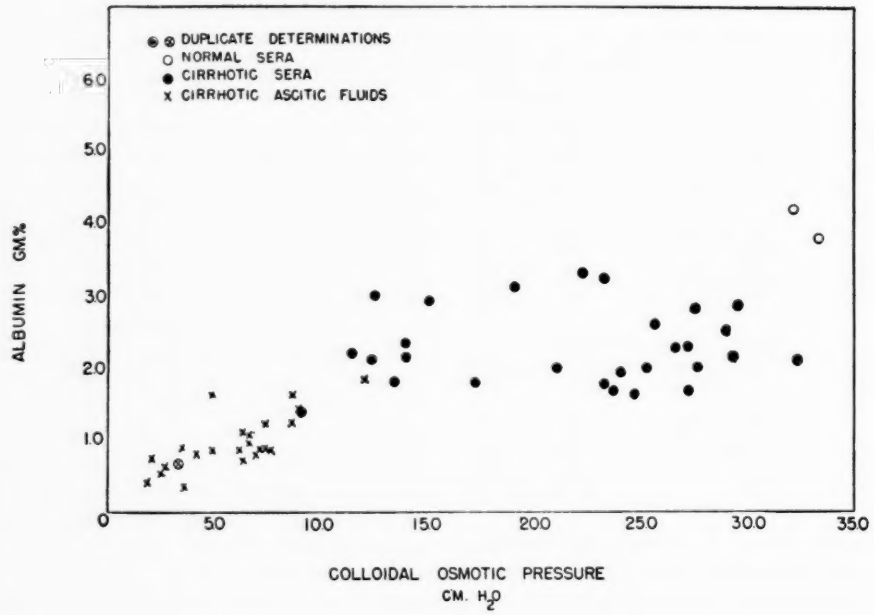


FIGURE 6

For description see text.

The problem thus posed is illustrated even more strikingly by the serial determinations of osmotic pressures, the serum albumin levels, and serum gamma globulin levels which are plotted against time for a single cirrhotic patient in Figure 7. This patient showed striking improvement on intensive dietary therapy over a period of half a year on our metabolic service. Toward the beginning of therapy (March-April) the serum osmotic pressures were consistently under 150 mm. of H_2O and the effective osmotic pressures (serum minus ascitic osmotic pressures) were in the neighborhood of 60 mm. of water, a range as low as that found in the severe nephrotic syndrome. After clinical improvement, *without any significant change* in the electrophoretically determined levels of albumin or gamma globulin, both serum and effective osmotic pressures have almost doubled.

Among the questions raised by these findings is this: Are there proteins present in the serum of the patient severely ill with cirrhosis which, though indistinguishable by electrophoresis from the proteins of remission, have a very much smaller effect on colloidal osmotic pressure? If so, at least two possible bases come to mind. The first, a grossly altered net charge, seems unlikely on the basis of indistinguishable electrophoretic behavior. The second, a higher average molecular weight, might exist on the basis of either (a) a complexing of molecular species at the protein concentrations found in serum (with dissociation of the complex on the dilution ordinarily used in electrophoresis), or (b) the actual existence of a considerable proportion of proteins with molecular weights higher than those ordinarily associated with the known components in normal serum (25, 37).

p/c *curves, average molecular weights, and the question of dissociable aggregates in the sera of nephrotic and cirrhotic patients*

Measurements of osmotic pressure of quantitative serial dilutions of sera are admirably adapted to the investigation of both the latter possibilities. The relation of osmotic pressure to concentration of serum proteins under physiological conditions can be represented by the following expression:

$$\left(\frac{p}{c}\right) = A + Bc$$

where p is osmotic pressure and c is protein concentration (38).

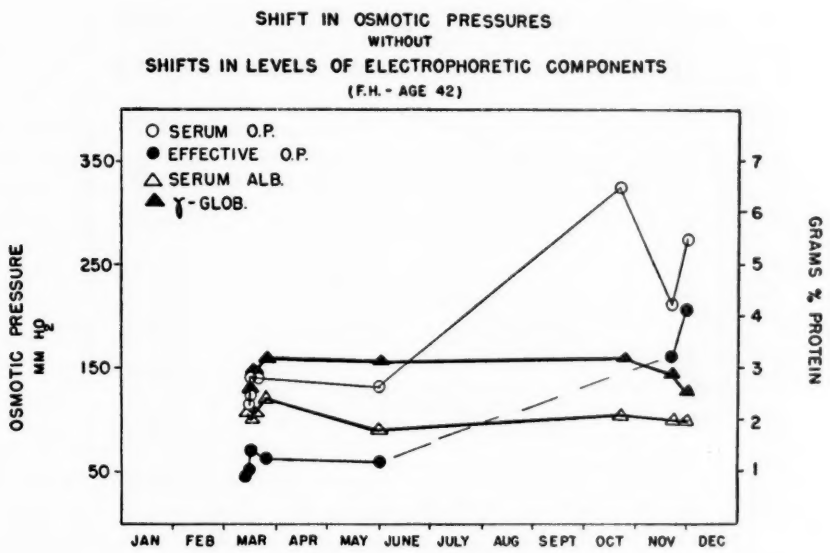


FIGURE 7

For description see text.

A (the intercept and a constant for a given mixture) is linear in the reciprocal of the average molecular weight at high dilutions; thus the extrapolation of a p/c *vs.* c plot to infinite dilution gives a direct estimate of average molecular weights.

B (the slope) is as a rule independent of protein concentration in the range usually studied and is related to the interactions of the protein molecules with each other and with salts. For most protein fractions prepared from sera of normal subjects, the slope B plot has proved positive in sign, though variable in magnitude according to the proteins and the conditions of study (25).

The literature on p/c plots of sera from patients with certain disease states is sparse. Wells, Youmans, and Miller (21) were well aware of the usual

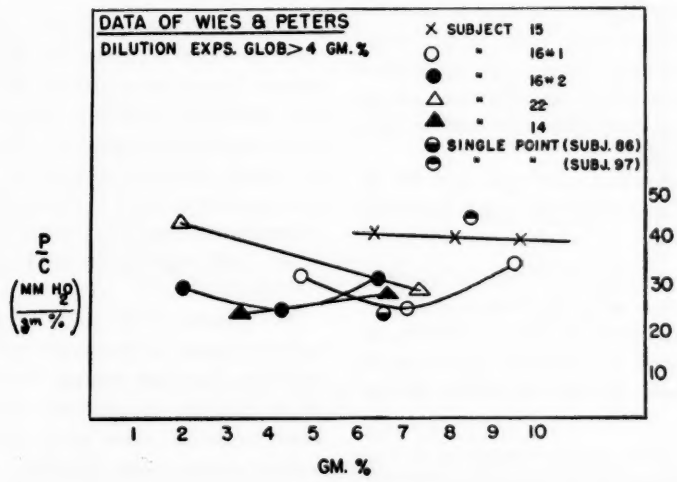


FIGURE 8

For description see text.

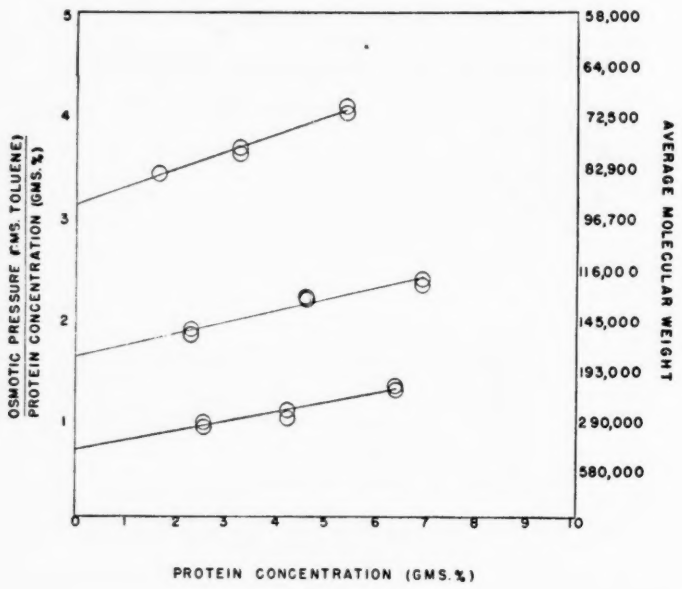


FIGURE 9

For description see text.

positive slope in their early publication. In Figure 8 are given the p/c plots made from tabulated data of Wies and Peters (22) where globulin levels have been greater than 4 Gm. per cent. The curves drawn between their points *suggest* occasional negative slopes. This suggestion (subject to high demands of accuracy of measurement for this type of plot) is of unusual interest for this reason. A negative slope in the right-hand (concentrated) segments of the plots which either flatten or turn positive as concentrations approach zero, gives indication of the dissociation of a large molecule aggregate into component smaller units with increasing dilution.

We, therefore, renewed investigation of the intercepts and the slopes of p/c *vs.* c plots in the following situations:

a) single serum specimens from five cirrhotic patients chosen for severity of edema and ascites;

b) single serum specimen from a cirrhotic patient chosen because of extreme hyper-gamma globulinemia despite absence of edema and ascites;[5]

c) twelve sera and three preparations of unfractionated urinary protein from eight patients in various stages of the nephrotic syndrome;

d) as controls we studied the proteins of sera of the normal male and of samplings from preparations of normal human serum albumin for pyrogen testing by the Commission on Plasma Fractionation.

In Figure 9 are plotted experimental points (in duplicate when available) for plasma from normal patients (top), for a patient with nephrosis in recent remission (middle), and for a patient with severe nephrosis (bottom). The average molecular weights corresponding to the intercepts on the left are given at right of the figure. The value for unfractionated normal plasma proteins (95,000) is in good agreement with previous findings (90,000) (27).

The proteins of the serum of the patient in the nephrotic phase of glomerulonephritis in moderate remission have an average molecular weight of about 160,000; the proteins of the patient in the severe nephrotic phase have an average molecular weight greater than 400,000. The range (between 160,000 and 830,000) of the sera from the

[5] We are indebted to Dr. Murray Franklin of the Cook County Hospital, Chicago, Illinois, for making these sera available for study.

nephrotic patients is consistent with the known presence of large quantities of β-lipoproteins (of molecular weight greater than 1,000,000 when separated from normal plasma [38]). That little of this high molecular weight protein gets into the urine is further confirmed by a maximum average molecular weight of 84,000 for the unfractionated urinary proteins recovered from our severest nephrotic patient. (Molecular weight of serum albumin from a normal patient is 69,000 [27]; several samples of the product available for intravenous human use give an average molecular weight of 73,000 in our hands.)

For all sera of nephrotic patients and for the urinary proteins, the slopes (B) of all the p/c plots (comparable to those of Figure 9) have been positive, though for sera showing high average molecular weights the magnitude of the slopes has been somewhat less than that seen in either sera of normal subjects or urinary proteins.[6] This indicates that in sera of nephrotic patients, as for the β-lipoproteins separable from sera of normal patients, there is no dissociation of this type of macromolecule with dilution.[7]

The p/c plot (together with the total protein level and electrophoretic distributions) for the cirrhotic patient with extreme hyper-gamma globulinemia is illustrated in Figure 10 and is representative of our results with the cirrhotic group. In this patient the average molecular weight (135,000) is not very different from that (140,000) expected for a mixture of eighteen parts of molecular weight 69,000 and 65 parts of 160,000 (this ignores the 17 parts contributed by the alpha and beta globulins in the electrophoretic diagrams). This value, however, is the largest average molecular weight encountered in six cirrhotic patients so studied. For the five water-logged cirrhotic patients, average molecular weights lay between this and the figure for the normal patients. Though values predicted from albumin and gamma globulin levels were consistently greater than those measured, average molecular weights were never sufficiently great to yield an explanation of findings for the patient illustrated in Figure 7 in terms of the presence of large quantities of macromolecules of

[6] This finding is consistent with the small positive slopes previously noted for crude preparations of normal β-lipoproteins.

[7] In this series of experiments we endeavored to compare immunochemically determined albumin levels with uncorrected electrophoretic albumin levels in order to get an estimate (using the normal molecular weights for the albumin and gamma globulin components) of the average molecular weights of the combined beta and alpha globulins. However, because we encountered sufficient difficulty with the optical measurement of end points in immunochemical titrations of these sera, we consider the comparison and subsequent calculations unreliable.

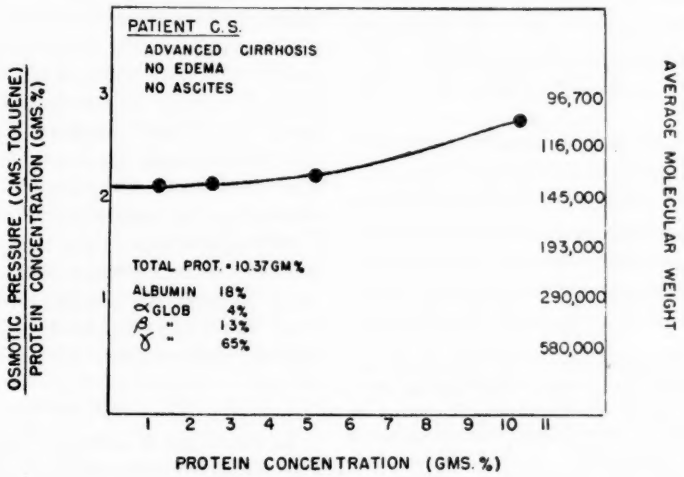


FIGURE 10

For description see text.

size range comparable to those encountered in the nephrotic syndrome.

Likewise, the slopes of the p/c plots of the cirrhotic group were consistently positive, thus yielding no explanation in terms of dissociation of macromolecules with dilution. Unfortunately, at the time this phase of our study was undertaken no early sera from the patient of Figure 7 remained.

DISCUSSION

The data here presented give clear indication that a comparatively recent empirical relation between osmotic pressure and electrophoretically determined albumin and globulin levels may not fit the data in the instance of the hypoproteinemia of various stages of the nephrotic syndrome as well as do older relations based on salting out determinations even though laborious refractometric corrections are carried out (39).

The situation for uncorrected electrophoretic data in Laennec's cirrhosis seems little better.

Taken in the light of the known heterogeneity, from the standpoint of size, shape, and net charge, of the proteins which in an electric field migrate in one component, these results are not surprising. Thus, for example, Oncley, Scatchard, and Brown (25) show that the alpha globulin component of serum from normal subjects contains a protein much closer in molecular weight to the normal serum albumins than to the normal gamma globulins. On the other hand, the lipid rich component of alpha-2-globulin has a molecular weight higher than the average molecular weight for serum gamma globulin.

If there is this much heterogeneity in the proteins of plasma from normal subjects, so, also, during the course of the clinical hypoproteinemias, there may well be shifts in the various molecular species of protein responsible for osmotic pressure (40, 41) without comparable shifts in either gross salting out or electrophoretic distributions.[8]

It is perhaps disappointing that the intercepts and slopes of the p/c *vs.* c plots have not yielded the basis for the shifts of pressure without comparable electrophoretic shifts which we have seen clinically. We do plan to continue this exploration, particularly on serial sera of cirrhotic patients with very low osmotic pressures at the initiation of study. For it is possible that such sera will yield sufficient discrepancy between expected and measured average molecular weights; or between expected and measured slopes of the p/c plot, to warrant fractionation procedures aimed at purifications of components responsible for unusual osmotic properties.

It is, however, our feeling on the basis of studies here reported that in experimental work aiming to relate forces governing either equilibrium, or a steady state of flow, of water across a capillary membrane, the wisest course in hypoproteinemic conditions is to measure the osmotic pressure directly until such time as there has been accumulated a detailed knowledge of the size and charge of the various proteins encountered in each clinical condition associated with hypoproteinemia. Direct measurements of osmotic pressure, on a set of osmometers simultaneously operated in a single thermostat bath, can be carried out without undue expense of time.

Thus we have not extended our own electrophoretic-refractive increment-osmotic pressure measurements to a larger series and tried to fit other empirical equations, for it has seemed to us *a priori* unlikely that the same empirical equation can be used with sufficient accuracy on individual sera of varying hypoproteinemic etiologies. It takes a very small pressure gradient to move a great deal of water, given a big enough path; therefore, the accurate quantitation of small gradients is essential in the analysis of mechanism.

The significance of the time relations of these and other osmotic pressure measurements to movements of water in nephrotic, cardiac, cirrhotic, and other water-logged patients will be discussed in subsequent papers from this group.

SUMMARY

In the course of a series of direct osmotic pressure measurements on individual sera of nephrotic

[8] In studies on albumin metabolism in hypoproteinemic states, where albumin levels, rather than osmotic pressures, become of interest, the electrophoretic method as modified by corrections for refractive increments has a real advantage in reproducibility over solubility methods in the presence of very low albumin levels. Quite frequently, clinicians have sent to us sera of nephrotic patients with hypoproteinemia which have aroused interest by anomalously high albumin-globulin ratios in hospital laboratories. These sera on electrophoresis have invariably shown the characteristic pattern of the nephrotic state.

children undergoing spontaneous diuresis induced by measles, simultaneous determinations of protein nitrogen, electrophoretic distribution, and protein specific refractive increments have been carried out on a small group of sera, in order to see whether, with the most accurate possible use of the electrophoretic technique, a recently proposed relation for osmotic pressure of normal pooled plasma in terms of albumin and globulin levels can be applied. The results indicate that the relation gives a good correlation with measured pressures in the low pressure range, but in the intermediate range the predicted pressures are much lower than the measured and the discrepancies much larger than the reproducibility of the osmotic pressure method.

In a series of sera and ascitic fluids of cirrhotic patients studied by uncorrected electrophoresis, predictability of measured osmotic pressures proved not much better.

In serial sera from a patient with severe cirrhosis showing clinical improvement, osmotic pressure doubled without significant shifts in serum levels of electrophoretic components.

No evidence for either significant quantities of unusually large non-lipid bearing globulin molecules, nor of dissociation of larger complexes with dilution, could be obtained from intercepts or slopes of $\frac{\text{pressure}}{\text{concentration}}$ *vs.* concentration plots in a small group of water-logged cirrhotic patients. Average molecular weights predicted from normal weights of electrophoretic albumins and gamma globulins, together with the distributions for certain disease states, proved only slightly higher than measured average molecular weights of the proteins of these sera.

Similar plots of sera from nephrotic patients gave evidence of high average molecular weights (range 160,000 to 830,000) varying with the stage of the syndrome. There was no evidence of dissociation of such aggregates with dilution.

These plots have not disclosed, but do not rule out, the existence of proteins of unusual osmotic properties as suggested by osmotic pressure measurements on certain patients with severe hypoproteinemia and edema.

It is concluded that in the extreme states of hypoproteinemia, where accurate balance between osmotic forces is needed for representation of the dynamics, direct measurements cannot be obviated.

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THE ELECTROPHORETIC DISTRIBUTION OF PROTEINS IN PLASMA IN RHEUMATOID ARTHRITIS [1, 2]

By M. W. ROPES, G. E. PERLMANN,[3] D. KAUFMAN, AND W. BAUER

(From the Departments of Medicine of the Massachusetts General Hospital, and Harvard Medical School, Boston, Mass.)

(Submitted for publication August 4, 1953; accepted September 23, 1953)

Abnormalities in the electrophoretic patterns of plasma proteins in rheumatoid arthritis have been described by various workers. With few exceptions an increase in the relative concentration of α_1-, α_2-, and γ-globulin and of fibrinogen and lowering of the albumin concentration have been observed and have been associated consistently with activity of the disease (1–15). Olhagen (4, 10) and Svartz and Olhagen (8) ascribed a possible prognostic significance to isolated elevation of α- or γ-globulin. They suggested that an elevation of the α-globulins without an increase in the γ-fraction, often noted in acute cases, indicated a tendency to more rapid healing, whereas a high γ-globulin with low α-globulins represented a poor prognosis. Wallis (13) described a "T component" in three patients with active rheumatoid arthritis. A protein with a similar electrophoretic mobility, γ'-globulin, was noted by Paul and Routh (14) in normal sera. These authors observed a small decrease of this fraction in the sera of seven patients with rheumatoid spondylitis but found no change in 27 patients with generalized rheumatoid arthritis. Recently, Laurell (16) and Mehl and Golden (17) have demonstrated the occurrence of small amounts of two proteins with isoelectric pH values lower than that of serum albumin when electrophoresis was performed in acetate buffer of pH 4.5. The concentration of one component, which at pH 8.6 in diethylbarbiturate migrates with a mobility of the α_2-globulin, was considerably increased in sera from patients with rheumatoid arthritis (17) and disappeared on treatment with cortisone (16).

In the present series the electrophoretic patterns of 88 plasmas from 32 patients with rheumatoid arthritis have been studied. The changes in the protein fractions were correlated with the duration of the disease and with the clinical and laboratory indications of the degree of severity of the arthritis. Classification as to stage of disease and functional ability was made by the criteria approved by the American Rheumatism Association (18).[4] Alterations in the protein fractions have been followed during periods of exacerbations and remissions of activity of the arthritis. Of the 32 patients, 4 had had rheumatoid arthritis for less than one year, 7 for one to two years, 5 for two to five years, 8 for five to ten years, and 8 for over ten years.

EXPERIMENTAL TECHNIQUES

Blood for analysis was collected in the fasting state with 2 mg. potassium oxalate per ml. as an anticoagulant. After removal of the red cells by centrifugation, the plasma was stored at $-30°$ C. until analyzed. The Pregl micro-Kjeldahl method was used for the determination of total nitrogen, and of the non-protein nitrogen on a tungstic acid filtrate. Prior to electrophoresis the plasma was diluted with buffer to a nitrogen concentration of 3.0 mg. per ml., corresponding to 1.87 per cent protein, assuming a factor of 6.25 for conversion to weight basis. The solution was then dialyzed at 5° C. for three days with frequent changes against large volumes of the buffer employed in the analysis. In most experiments a 0.1 N sodium diethylbarbiturate buffer of pH 8.6 and 0.1 ionic strength was used, but

[1] This is publication No. 151 of the Robert W. Lovett Memorial for the Study of Crippling Disease, Harvard Medical School.

[2] The expenses of the investigation were defrayed in large part by a grant from the Commonwealth Fund.

[3] Now at the Rockefeller Institute, New York.

[4] The stage of disease is determined by the degree of presumably irreversible involvement rather than activity. Stage I is characterized only by subchondral bone atrophy; Stage II by cartilaginous and cortical destruction; Stage III by deformities such as ulnar deviation and subluxation; Stage IV by fibrous or bony ankylosis. Nodules and significant muscle atrophy must not be present in Stage I. The functional classes are: I. Ability to carry on all usual duties without handicaps; II. Adequate for normal activities despite handicap of discomfort or limited motion in one or more joints; III. Limited only to few or none of the duties of usual occupation or self-care; IV. Incapacitated, largely or wholly; little or no self-care.

TABLE I

The average electrophoretic distribution of proteins in six pools of normal human plasma *

Buffer composition	pH	Concentrations in per cent as					
		Albumin	Globulins				
			α_1	α_2	β	ϕ	γ
0.1N NaV–0.02 NHV†	8.6	56.5	4.9	8.3	13.7	5.2	11.4
0.064M K_2HPO_4–0.008 MKH_2PO_4	7.7	61.4‡		6.4	15.0	5.3	11.9

* Protein concentrations: 1.8 per cent.
† V = diethylbarbiturate.
‡ Albumin and α_1-globulin unresolved in this solvent.

in a few cases the solvent was potassium phosphate of pH 7.7 and 0.2 ionic strength. Electrophoresis was performed at 1.5° C. in the apparatus described by Tiselius (19), equipped with the Schlieren scanning device of Longsworth (20).

The concentrations of the electrophoretic components were calculated from planimetric measurements of the enlarged tracings of the diagrams. The method of Tiselius and Kabat (21) was used to allocate the area of each peak. The results are expressed in per cent, *i.e.*, the ratio of the area ascribed to each component to the total area, exclusive of the δ and ϵ boundaries. Each value is the average obtained from the ascending and the descending pattern.

Blood for the determination of sedimentation rates by the method of Rourke and Ernstene (22) was collected on the same day as that for electrophoresis, with 1 mg. heparin (Hynson, Wescott, and Dunning) per 4 ml. of blood as anticoagulant.

Fibrinogen was estimated according to the method of Cullen and Van Slyke (23).

RESULTS

As shown by Svensson (24, 25) and also in this laboratory (26), the relative distribution of electrophoretic components is a function of the protein concentration and the ionic strength. Therefore, the experiments reported here were all carried out with a protein concentration of 1.87 per cent and at constant ionic strength. As a basis for comparison the values obtained from six pools of normal plasma in the two solvents (Table I) were used. The difference in the apparent electrophoretic composition in the two types of buffers is slight, and the results are in close agreement with those of other investigators (27–33).

The influence of fibrinogen on the distribution of the protein fraction is evident from a comparison of the values obtained with serum, defibrinated plasma, and plasma (Table II). The concentration of fibrinogen calculated from the electrophoretic patterns of normal plasma and from those of rheu-

TABLE II

Electrophoretic distribution of proteins in normal serum, plasma and defibrinated plasma * †

Case	Material	Total protein *Gm./100 cc.*	Concentrations in per cent as					
			Albumin	Globulins				
				α_1	α_2	β	ϕ	γ
1	Serum		61.9	3.8	5.8	14.2		14.3
	Defibrinated plasma		61.3	4.0	5.9	14.8		14.0
	Plasma		58.2	4.1	5.9	14.7	5.9	11.2
2	Serum	7.62	59.6	4.3	9.6	12.1		14.4
	Defibrinated plasma		59.0	4.3	9.9	12.8		14.0
	Plasma	7.05	55.2	4.8	9.7	12.8	5.4	12.1
3	Serum		59.7	3.2	9.0	13.2		14.9
	Defibrinated plasma		59.5	3.3	8.3	14.0		14.9
	Plasma	6.69	57.9	3.4	8.3	12.7	5.6	12.1
4	Serum		57.0	5.3	11.0	13.7		13.0
	Defibrinated plasma		57.2	5.2	10.5	14.0		13.1
	Plasma	6.75	52.5	5.3	10.6	14.3	7.2	10.1
5	Serum	7.75	58.7	4.5	7.6	14.1		15.1
	Defibrinated plasma		58.3	4.2	7.1	14.4		16.0
	Plasma	6.94	56.7	4.1	7.3	12.5	7.4	12.0
6	Serum	6.19	55.6	4.4	11.9	16.3		11.8
	Defibrinated plasma		54.8	4.5	12.3	16.7		11.7
	Plasma	5.80						

* Protein concentrations: 1.8 per cent.
† In sodium diethylbarbiturate buffer of pH 8.6 and 0.1 ionic strength.

TABLE III

Variations in the electrophoretic distribution of proteins in rheumatoid plasma * †

Unit No.	Total protein *Gm./100 cc.*	Concentrations in per cent as						Fibrinogen (chemically) Per cent of total protein	Sedimentation rate *mm./min.*
		Albumin	Globulins						
			α_1	α_2	β	ϕ	γ		
Normal	7.00	56.5	4.9	8.3	13.7	5.2	11.4	3.6	0.40
393050	7.07	50.0	8.2	15.1	12.1	6.0	8.6	6.4	1.00
379668	5.19	47.9	6.6	12.6	13.7	10.1	9.1	7.4	1.24
425859	6.81	41.6	6.5	10.4	14.5	15.2	11.8	9.3	1.62
352223	6.87	45.5	6.4	11.9	13.6	9.7	12.9		1.56
631712	7.50	48.6	6.2	10.3	11.6	7.2	16.1		0.73
386915	6.63	48.8	4.3	7.5	14.6	8.0	16.8	4.3	0.60
350995	7.06	47.5	5.8	9.9	11.1	7.4	18.3	5.5	1.19
389427	6.94	45.6	6.1	9.7	11.9	8.5	18.2	6.4	0.69
327699	7.16	36.9	6.9	13.0	12.9	11.2	19.2	8.8	1.66
169863	7.38	42.7	5.4	7.5	12.3	11.8	20.3	4.8	1.44
519333	6.75	37.2	7.0	13.1	12.5	9.8	20.4	7.6	1.52
389209	7.87	30.5	7.2	11.7	10.6	11.5	28.5		1.52

* Protein concentrations: 1.8 per cent.
† In sodium diethylbarbiturate buffer of pH 8.6 and 0.1 ionic strength.

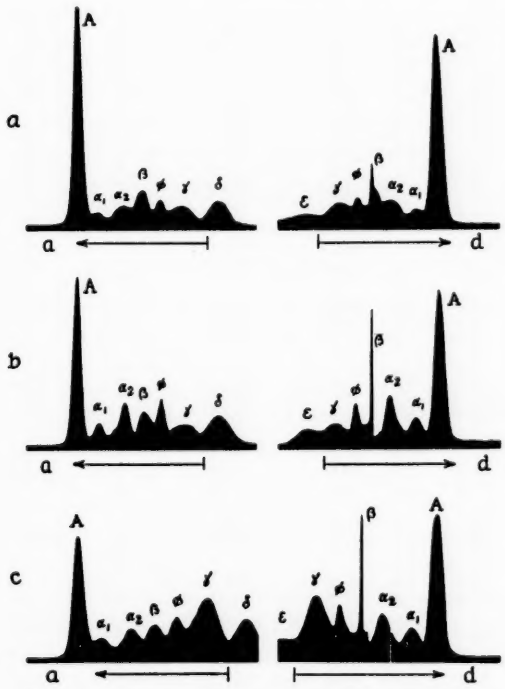

FIG. 1a, b, AND c. ELECTROPHORETIC PATTERNS OF 1.8 PER CENT SOLUTIONS OF PROTEINS OF NORMAL PLASMA (a) AND OF THE PLASMA OF PATIENTS WITH RHEUMATOID ARTHRITIS (b AND c)

Electrophoresis was carried out in sodium diethylbarbiturate buffer of pH 8.6 and $\Gamma/2$ 0.1 for 12,600 seconds at a potential gradient of 5.1 volts per centimeter.

matoid arthritis was always 1.5 to 2-fold higher than the chemically determined value. This may be taken as an indication that the electrophoretic fraction is increased by some non-clottable protein with a mobility similar to that of fibrinogen.

Characteristic plasma electrophoretic patterns in active rheumatoid arthritis are well exemplified in Figure 1, which for comparison also shows that of normal plasma. The extreme variation from high concentrations of α_1- and α_2-globulins, associated with normal γ-globulin (Figure 1b) to marked elevation of the γ-fraction in the presence of only a slight abnormality of the α-globulins (Figure 1c) is demonstrated. Further evidence of the variations in the concentrations of individual fractions which occur in rheumatoid arthritis is provided in Table III. In general, all of the globulins and the fibrinogen are elevated, but in the majority the greatest change is in the γ-globulin fraction, which occasionally rises to 32 per cent. The albumin concentration may decrease to 30 per cent.

Consecutive observations of the electrophoretic distributions of plasma proteins during the course of exacerbations and remissions in rheumatoid arthritis indicate that the protein changes reflect alterations in clinical activity of the disease and correlate, in general, with variations in the sedimentation rate. Elevation of the α-globulins apparently tends to occur early in the course of increasing activity of the disease and is followed by a predomi-

nance of γ-globulin in later stages. (See Case 1 in 1942 and in 1950, and Case 4, Table IV.) These findings are similar to those described by Olhagen (4, 10) and Dole and Rothbard (9) in rheumatoid arthritis and those noted in the course of other diseases (35–37).

In most cases, however, shifts in the relative concentrations of albumin, globulin, and fibrinogen occur simultaneously and correspond to alterations in the clinical state and sedimentation rate (see Cases 1 through 4, Table IV). Occasionally the fibrinogen concentration remains unaltered despite a marked reduction in the sedimentation rate (see Cases 5 and 6).

Dissociation of the sedimentation rate from the clinical and electrophoretic manifestations of disease activity was found in Case 7 of Table IV. During a period of several years (from 1943 to 1947) in which the clinical course and the sedimentation rate remained relatively constant, the composition of the electrophoretic pattern did not change. On a subsequent severe exacerbation (in

TABLE IV

Changes in the electrophoretic distribution of plasma proteins with alterations in activity of rheumatoid arthritis *

	Case	Date	Concentrations in per cent as						Sedimentation rate *mm./min.*	Stage of disease¶	Functional class¶
			Albumin	Globulins							
				α_1	α_2	β	ϕ	γ			
	Normal		56.5	4.9	8.3	13.7	5.2	11.4	0.40		
1	J. A.†	12/17/42	44.5	7.6	15.8	11.5	9.3	11.3	0.94	3	4
	384135	2/16/43	42.1	7.5	12.8	13.1	8.8	15.7	1.10	3	4
		5/11/44	51.4	5.6	11.1	12.7	6.7	12.5	1.32	3	3
		10/19/44	59.5	4.6	9.5	12.7	5.9	7.8	0.33	3	2
		6/13/46	46.0	6.9	13.3	11.9	10.9	11.0	1.24	3	4
		1/21/47	46.7	7.0	12.3	12.4	7.6	14.0	0.68	3	4
		1/15/48	48.0	6.3	10.8	14.5	8.5	11.9	0.84	3	4
		2/17/49	44.3	7.1	12.4	13.0	9.3	13.9	1.12	3	4
		7/20/49‡	47.8	6.5	12.2	15.9		17.6	1.56	3	4
		9/1/49‡§	55.9	5.1	11.2	13.3		14.5	0.15	3	2
		12/24/49‡§	57.0	6.7	12.7	16.6		7.0	0.14	3	2
		1/4/50‡	49.5	8.3	15.8	18.5		7.9	1.24	3	4
		1/20/50‡	47.6	6.5	12.5	17.5		15.9	1.26	3	4
		3/2/50‡	56.8	5.4	10.1	14.3		13.4	0.70	3	3
2	A. P.	12/19/46	42.1	7.6	5.3	11.9	8.5	24.6	1.22	3	2
	216602	3/6/47	45.9	7.5	8.3	8.7	9.7	19.9	0.86	3	2
		12/11/47	50.7	8.6	6.2	10.5	6.4	17.6	0.43	3	1
3	W. M.	4/22/47	46.1	5.3	13.3	14.7	6.9	13.7	0.84	1	4
	567613	1/8/48	57.3	4.4	12.9	13.9	5.5	6.0	0.18	1	2
		12/16/48	53.5	5.1	13.4	14.0	6.5	7.5	0.42	1	1
4	G. R.	10/21/42‖	47.2		12.6	11.9	12.8	15.5	0.67	1	2
	319835	9/28/32	46.2	4.9	9.6	13.1	6.6	19.6	1.00	1	2
		6/1/44	59.7	5.3	7.5	11.4	5.7	10.4	0.30	1	1
5	E. B.	4/11/44	48.6	5.7	10.4	13.7	6.5	15.1	1.05	1	2
		1/28/47	54.0	4.4	4.9	17.2	6.6	12.9	0.26	1	1
6	E. H.	7/27/43	42.5	6.4	9.3	14.4	7.6	19.8	1.09	2	3
	311289	1/8/48	50.7	4.9	9.0	14.2	7.5	13.7	0.62	1	1
7	A. M.	8/12/43	46.7	7.3	15.1	10.9	9.2	10.8	1.00	2	3
	393050	6/22/44	42.1	7.8	15.3	11.4	10.3	13.1	0.79	2	3
		3/1/45	45.6	7.2	14.0	11.4	8.9	12.9	1.24	2	2
		8/1/46	42.0	7.8	15.7	11.7	10.6	12.2	1.45	2	3
		2/14/47	45.5	7.3	15.3	10.2	9.2	12.5	1.18	2	3
		12/6/49	33.7	9.0	17.1	10.5	13.1	16.6	1.93	3	4
		7/6/50	44.4	6.6	13.4	11.2	9.8	14.6	1.72	3	3

* Protein concentrations: 1.8 per cent; in sodium diethylbarbiturate buffer of pH 8.6 and 0.1 ionic strength.
† Previously reported (34).
‡ Electrophoresis carried out on serum.
§ Patient treated with ACTH.
‖ In potassium phosphate buffer.
¶ See footnote 4 to text.

1949) a marked alteration in the electrophoretic pattern was associated with a rise in the sedimentation rate. A return toward a more normal electrophoretic pattern and a considerable improvement clinically occurred in 1950, with relatively little change in the sedimentation rate. In two other cases, not listed in Table IV, in which only sera were analyzed, a similar dissociation was found. The serum pattern reflected decreasing activity of the disease in one case during 1945 and 1946, and subsequently reached its most abnormal level after an exacerbation in 1948. Throughout this period the sedimentation rate varied only from 1.2 to 1.7 mm. per minute and was the same, 1.7 mm. per minute, both at the time of the partial remission in 1946 and during the exacerbation in 1948. Decreasing activity of the disease in 1948 in the other case was associated with a return toward a normal electrophoretic pattern, but the sedimentation rate continued to rise at this time.

Clinical remission of rheumatoid arthritis, whether spontaneous or induced by ACTH or cortisone, is usually associated with lessening of the abnormalities in the electrophoretic pattern. A return to a normal distribution of the components, occasionally with the relative concentration of the γ-globulin somewhat below the normal concentration, may occur when the remission is relatively complete (see Cases 1, 4, and 5, Table IV). Similar observations have been reported by other workers in rheumatoid arthritis (9, 12, 15, 38, 39), as well as in rheumatic fever (7, 35, 40), disseminated lupus erythematosus (41, 42), and scleroderma (42).

Numerous attempts have been made to correlate alterations in the sedimentation rate with changes in the concentration of individual plasma protein fractions. A major role has been ascribed to elevations of fibrinogen or of the α_1-, α_2-, or γ-globulin, respectively (4, 33, 43–45). In the present study a correlation between the sedimentation rate and high concentrations of fibrinogen and γ-globulin and low concentration of albumin is apparent with coefficients of .61, .60, and − .58, respectively.[5]

A relationship of the alterations in protein fractions to severity of the disease was apparent only in the case of the albumin and of the fibrinogen. With increasing severity the albumin concentration fell from an average of 50.1 per cent in the mild cases to 41.1 in severe cases. Concomitantly, there was a rise in fibrinogen from 6.9 per cent in the mild cases to 9.8 in the severe ones.

The electrophoretic patterns of plasma of patients whose disease is of varying duration indicate no definite correlation between any of the protein fractions and the duration of the disease. There is a slight tendency toward a higher γ-globulin, 19.9 per cent, in the patients of over ten years' duration, as compared with 15.7 per cent in patients whose disease is of less than one year.

DISCUSSION

The consistent finding in the present study that in cases of active rheumatoid arthritis the relative concentrations of α_1-, α_2-, γ-globulin and fibrinogen are increased and that of albumin decreased correlates well with those of previous reports. A tendency is apparent for the α-globulins to rise early in the disease followed by an elevation of the γ-globulins. The causes for the alterations in a given individual protein fraction, however, are unknown, although there is evidence that the α-globulins are increased whenever considerable inflammation or tissue destruction is present and that a rise of γ-globulin may be associated with enhanced antibody formation. However, a direct correlation between protein variations and the pathogenic mechanisms of rheumatoid arthritis is not possible.

SUMMARY

The electrophoretic distribution of proteins in 88 plasmas from 32 patients with rheumatoid arthritis has been determined.

An elevation of the concentrations of α_1-, α_2-, and γ-globulins and of fibrinogen and a lowering of the albumin were observed in active rheumatoid arthritis.

Reversal to a normal electrophoretic pattern may occur during remission of the activity of arthritis.

ADDENDUM

Electrophoretic Distribution of Proteins in Plasma in Other Rheumatic Diseases

Adequate comparison of the electrophoretic distribution of plasma proteins in rheumatoid arthritis

[5] The "correlation coefficient" is a measure of the degree of linear relationship between two quantitative variables. The coefficient can range in value from − 1 to + 1, the two extremes indicating, respectively, an exact inverse and an exact direct linear relationship.

with that in other joint diseases cannot be made because of the paucity of studies of these other diseases. Some interesting similarities and dissimilarities, however, are disclosed by the few studies available.

In plasma from seven patients with disseminated lupus erythematosus studied in this laboratory, the changes in the α-globulins were less than those noted in rheumatoid arthritis. In two cases the α_1-globulins were slightly elevated, and in one case 14.5 per cent α_2-globulin was recorded. One patient on two occasions had increased β-globulins, 21 and 23 per cent, respectively. The elevation of fibrinogen and γ-globulin was similar to that found in rheumatoid arthritis except for the fact that in two cases the levels of γ-globulin, 39 and 45 per cent, were greater than any observed in our series of rheumatoid patients. The albumin contents in these two cases were 34 and 24 per cent. Coburn and Moore (46) in two patients, Walker and Benditt (47) in nine, and Lever, Schultz, and Hurley (42) in nine noted similar patterns with slight or no abnormality in the α-globulin fractions and slight to marked elevation of γ-globulin. The distribution found by Reiner (41) in five cases was similar except for considerable increase in α_2-globulin.

Electrophoretic studies of plasma from four patients with mild to moderate dermatomyositis showed no elevation of the α_1-globulin and only a slight increase in the α_2-globulin and fibrinogen. In two patients the γ-globulin was normal; in the other two, however, it was increased to 19 and 20 per cent, respectively, and accompanied by a simultaneous decrease of albumin to 41 and 49 per cent. Similarly, in four advanced cases, Walker and Benditt (47) found only slight changes in the globulin fractions, in contrast to the usual finding in rheumatoid arthritis, but noted a moderate decrease in the albumin concentration. Lever, Schultz, and Hurley (42) reported similar changes in two severely ill patients but in another found marked increase in α_1- and α_2-globulin.

In rheumatic fever the findings in the three plasmas analyzed in this laboratory correspond to those reported by other investigators (7, 8, 15, 35, 47–51). In all cases a moderate to marked decrease in albumin concentration was associated with an increase in the α- and γ-globulin fractions. In some cases the α_2-globulin was much higher than that found in rheumatoid arthritis, and in others a marked rise in γ-globulin was observed; occasionally only a slight elevation in either the α- or γ-globulin was found. Wilson and Lubschez (51) concluded that the γ-globulin was elevated only when there had been an antecedent illness. Svartz and Olhagen (8) found high γ-globulin levels in recurrent cases or in cases manifesting involvement of organs other than joints.

In the serum from one patient with gout, the α-globulins were slightly increased, the γ-globulin fraction moderately increased, and the albumin concentration moderately lowered. This pattern resembled many of those found in rheumatoid arthritis.

Two sera from patients with Reiter's syndrome showed a considerable elevation in the α_1- and α_2-globulin fractions with only a slight elevation of γ-globulin and a moderate decrease in albumin. The level of the α-globulin components was higher, whereas that of the γ-globulin was lower than those observed in the majority of the rheumatoid patients. The distribution patterns resembled those reported by Svartz and Olhagen (8).

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ANNOUNCEMENTS OF MEETINGS

The 46th Annual Meeting of the American Society for Clinical Investigation will be held in Atlantic City, N. J., on Monday, May 3, 1954, with headquarters at the Chalfonte-Haddon Hall. The scientific session will begin at 9 a.m. at the Steel Pier Theater.

The annual meeting of the Association of American Physicians will be held at the Chalfonte-Haddon Hall on Tuesday, May 4, and Wednesday, May 5, 1954.

THE ELECTROPHORETIC PATTERNS OF PROTEINS IN SYNOVIAL FLUID AND SERUM IN RHEUMATOID ARTHRITIS [1, 2]

By G. E. PERLMANN,[3] M. W. ROPES, D. KAUFMAN, AND W. BAUER

(From the Departments of Medicine of the Massachusetts General Hospital and Harvard Medical School, Boston, Mass.)

(Submitted for publication August 4, 1953; accepted September 23, 1953)

The electrophoretic patterns of plasma obtained from patients with rheumatoid arthritis have been described by many investigators and are discussed in the preceding paper (1). The electrophoretic distribution of proteins in synovial fluid has been studied less extensively (2–5). It seemed of interest, therefore, to determine the various patterns which may occur in joint fluid of patients with rheumatoid arthritis and to determine whether a correlation exists with the duration and severity of the disease and with other characteristics of joint fluid which have been shown to reflect the degree of inflammation of the synovial tissue (6).

EXPERIMENTAL TECHNIQUES

A description of the technique employed in the aspiration of the synovial fluid from the knee as well as the methods used for the cell counts and for the characterization of mucin are adequately described in a recent publication (6). The procedures adopted for the electrophoretic and chemical analyses are identical with those given in the preceding paper of this series (1), with the exception that the synovial fluid was collected without an anticoagulant and is therefore compared with the corresponding serum.

RESULTS

Since normal human synovial fluid cannot be obtained in quantities sufficient for electrophoretic analysis, fluids from non-hemorrhagic effusions in traumatic arthritis [4] were taken as a basis of comparison.[5] As shown with the aid of the electrophoretic patterns of Figure 1a, traumatic fluids have five distinct fractions characterized by mobilities similar to those of the albumin, α_1-, α_2-, β- and γ-globulin of serum (cf. references 3 and 4). In addition, there is always present a component, denoted here as "Hy," which migrates with a velocity exceeding that of the albumin. Electrophoretic separation, followed by chemical analysis,[6] revealed that this substance is the polysaccharide, hyaluronic acid, which occurs in synovial fluid, and which has been studied electrophoretically also by Hesselvik (8) and Blix (9).

As illustrated in Table I, which records the electrophoretic composition of six traumatic synovial fluids, the apparent relative concentration of albumin is always higher in the fluid than in the corresponding serum, whereas that of the α_2-globulin is lower. A similar relationship of distribution of electrophoretic components to that of serum has been found previously in this laboratory in the analyses of joint fluid and lymph of cattle (7, 10).

In Table I are also given the electrophoretic mobilities of the different components which reveal a close agreement between the serum and the synovial fluid proteins. The value of -8.9×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1} recorded by us for hyaluronic acid is, however, lower than that reported by other workers (8, 9, 11). If in electrophoretic analysis a phosphate buffer of pH 7.7 and 0.2 ionic strength is substituted for the sodium diethylbarbiturate, the mobility of hyaluronic acid is about one unit more negative. A similar observation, *i.e.*, that a given solvent influences the migration of a polysaccharide or a mucoprotein to a greater extent than that of most proteins, has been made recently by Perlmann, Tamm, and Horsfall (12). This may

[1] This is publication No. 154 of the Robert W. Lovett Memorial for the Study of Crippling Disease, Harvard Medical School.

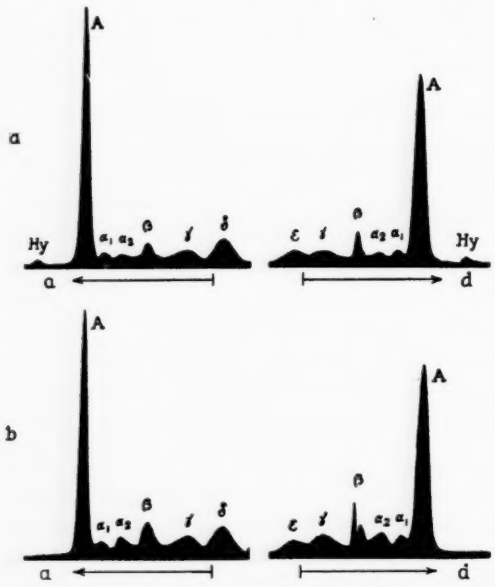
[2] The expenses of the investigation were defrayed in large part by a grant from the Commonwealth Fund.

[3] Now at the Rockefeller Institute, New York.

[4] We are indebted to Dr. James Heyl of Exeter, New Hampshire, for furnishing this material.

[5] Traumatic fluids have been found to show less change from normal in cells, sugar and protein content and state of mucin than fluid from any other type of joint disease (6).

[6] Electrophoretic separation and chemical analysis carried out on one sample of cattle fluid and one fluid from a patient with rheumatoid arthritis (7).



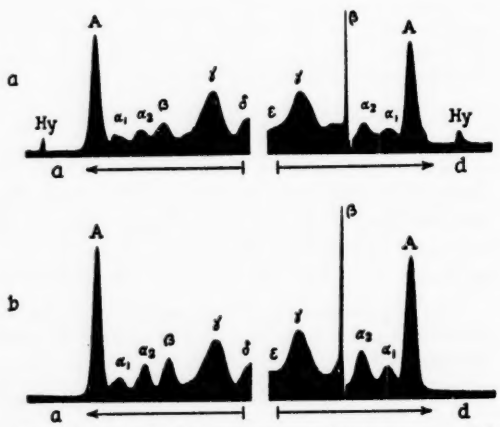
FIGS. 1a AND 1b. ELECTROPHORETIC PATTERNS OF A 1.8 PER CENT SOLUTION OF PROTEINS OF A TRAUMATIC SYNOVIAL FLUID (a) AND OF THE CORRESPONDING SERUM (b)

Electrophoresis was carried out in sodium diethylbarbiturate buffer of pH 8.6 and Γ/2 0.1 for 12,600 seconds at a potential gradient of 5.3 volts per centimeter.

explain, in part at least, the lower values recorded for hyaluronic acid in the present study.

Measurements were carried out on synovial fluids and sera of 21 patients with rheumatoid arthritis. Four of the patients had the disease for less than one year, two from one to two years, five from two to five years, and six from five to ten years and four over ten years. Classification as to stage of disease and functional ability was made by the criteria approved by the American Rheumatism Association (13).[7] In all, 31 fluids and 28 sera were studied. On the basis of the results of the electrophoretic analyses, the synovial fluids can be arranged in three distinct groups. As shown by representative cases listed in Table II, Group A contains fluids in which the apparent relative concentration of albumin is higher than in the corresponding serum, despite a wide range in concentration of albumin in the serum. In Group B are included those cases which have essentially the same relative distribution of electrophoretic components as the serum (see Figure 2); whereas, as illustrated with the aid of Figure 3, the fluids of Group C are characterized by a predominantly high concentration of γ-globulin. Two of the three cases studied by Svartz and Olhagen (3) apparently fall into Group A and the other into Group B.

Correlation between the electrophoretic pattern of joint fluids and the severity of disease is not good. Mild cases tend to show the type of pattern of Group A, with albumin concentrations higher in the fluid than in the serum. Of the ten cases in this category, four were in Stage I, three in Stage II, and three in Stage III. Of the cases in Group B, on the other hand, one was in Stage I, four in Stage II, two in Stage III, and one in Stage IV. However, despite this possible clinical correlation, no apparent connection exists between



FIGS. 2a AND 2b. ELECTROPHORETIC PATTERNS OF A 1.8 PER CENT SOLUTION OF PROTEINS OF THE SYNOVIAL FLUID (a) AND THE SERUM (b) OF A PATIENT WITH RHEUMATOID ARTHRITIS, REPRESENTATIVE OF GROUP B

Electrophoresis was carried out in sodium diethylbarbiturate buffer of pH 8.6 and Γ/2 0.1 for 14,400 seconds at a potential gradient of 5.1 volts per centimeter.

[7] The stage of disease is determined by the degree of presumably irreversible involvement rather than activity. Stage I is characterized only by subchondral bone atrophy; Stage II by cartilaginous and cortical destruction; Stage III by deformities such as ulnar deviation and subluxation; Stage IV by fibrous or bony ankylosis. Nodules and significant muscle atrophy must not be present in Stage I. The functional classes are: I. Ability to carry on all usual duties without handicaps; II. Adequate for normal activities despite handicap of discomfort or limited motion in one or more joints; III. Limited only to few or none of the duties of usual occupation or self-care; IV. Incapacitated, largely or wholly; little or no self-care.

TABLE I

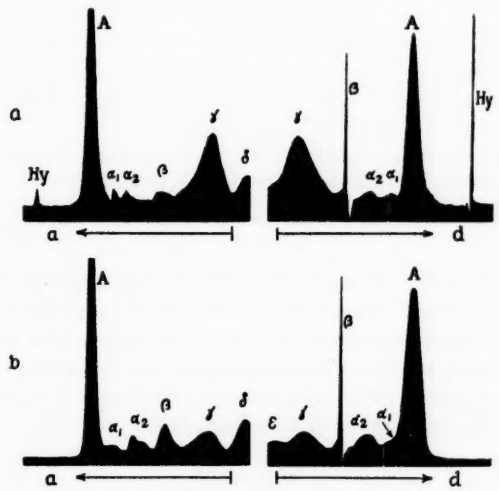
Electrophoretic distribution of proteins in serum and synovial fluid from traumatic arthritis *

Case		Total protein Gm./100 cc.	Concentrations in per cent as					Mobilities × 10^{-5} cm.^{2} volt.$^{-1}$ sec.$^{-1}$					
			Albumin	Globulins				"Hy"	Albumin	Globulins			
				α_1	α_2	β	γ			α_1	α_2	β	γ
A. B.	Fluid	2.99	61.5	5.5	5.5	12.9	14.6	−9.0	−6.6	−5.3	−4.4	−3.3	−1.3
	Serum	6.75	53.8	5.8	9.8	13.4	17.2		−6.5	−5.3	−4.0	−3.1	−1.2
M. C.	Fluid	3.57	64.2 *	3.3	7.4	11.1	14.0	−8.9	−6.2	−5.1	−4.1	−2.9	−1.2
	Serum	7.12	57.6	3.5	9.9	13.2	15.8		−6.2	−5.1	−4.1	−2.8	−1.2
J. C.	Fluid	3.54	64.6	4.5	5.8	12.3	12.8	−8.9	−6.4	−5.3	−4.4	−3.2	−1.5
	Serum	6.50	55.6	5.3	8.9	15.2	15.0		−6.4	−5.3	−4.2	−3.2	−1.4
P. M.	Fluid	3.45	70.3	4.1	5.1	8.9	11.6	−8.9	−6.3	−5.2	−4.1	−3.0	−1.2
	Serum	6.94	58.5	4.3	8.6	15.5	13.1		−6.5	−5.5	−4.3	−3.0	−1.3
L. O.	Fluid	3.90	65.2	4.1	7.7	12.5	10.5	−8.5	−6.0	−5.0	−4.0	−2.7	−1.2
	Serum	6.50	57.9	4.6	10.7	15.1	11.7		−6.3	−5.3	−4.2	−2.9	−1.4
C. S.	Fluid	3.08	64.2	5.2	6.9	14.3	9.4	−9.0	−6.4	−5.2	−4.4	−2.7	−1.4
	Serum	7.06	57.1	5.8	9.5	15.7	11.9		−6.2	−5.0	−4.0	−2.6	−1.1

* Protein concentrations: 1.8 per cent; in sodium diethylbarbiturate buffer of pH 8.6 and 0.1 ionic strength.

the erythrocyte sedimentation rate and the classification of patterns into Groups A and B, respectively.

A relationship between the electrophoretic distribution of protein fractions and the duration of effusion is suggested by the fact that five cases of Group A had effusions for less than eight months, which was the case for only one patient of Group B. Moreover, if effusion persisted for a longer period, an increase in the average concentration of γ-globulin became apparent. Thus, the relative concentration of this protein in fluids of less than one year was 17.5 per cent, compared with 46 per cent in those of five to ten years' duration.



FIGS. 3a AND 3b. ELECTROPHORETIC PATTERNS OF A 1.8 PER CENT SOLUTION OF PROTEINS OF THE SYNOVIAL FLUID (a) AND THE SERUM (b) OF A PATIENT WITH RHEUMATOID ARTHRITIS, REPRESENTATIVE OF GROUP C

Electrophoresis was carried out in sodium diethylbarbiturate buffer of pH 8.6 and $\Gamma/2$ 0.1 for 14,400 seconds at a potential gradient of 5.2 volts per centimeter.

The three patients included in Group C presented varied clinical pictures, ranging from mild to severe, with sedimentation rates of 0.14 to 1.2 mm. per minute and joint effusions lasting from 14 months to nine years. The only consistent finding in these three cases was that the arthritis in the knee from which the fluid was aspirated seemed of mild nature. Moreover, in two of the patients the changes in the other fluid findings (number of polymorphonuclear leukocytes, type of mucin precipitate and concentration of sugar) were also slight. The exceptionally low content of protein (3.2 per cent) in the one fluid of this type reported by Svartz and Olhagen (3) is of interest.

There is no apparent correlation in the three groups between the cell count, the character of the mucin, and the electrophoretic distribution of proteins. However, the average total protein concentration of the joint fluids of Group A, *i.e.*, 4.4 per cent, is lower than the 5.4 per cent found in Group B.

In one fluid the electrophoretic pattern disclosed

TABLE II

Electrophoretic distribution of proteins in serum and synovial fluid from patients with rheumatoid arthritis *

Unit No.		Total protein Gm./100 cc.	Concentrations in per cent as Albumin	Globulins α_1	α_2	β	γ	Joint fluid Leukocytes per cu. mm.	Polymorphonuclears %	Type of mucin ppt.	Stage of disease†	Functional classification†	Duration of effusion	Sedimentation rate mm./min.
								Group A						
311289	Fluid	3.98	51.5	6.6	8.6	11.6	21.7	10,000	54	—	2	3	2 yrs.	1.09
	Serum	7.56	41.0	7.5	10.0	15.4	26.1							
594438	Fluid	4.91	37.8	10.7	13.4	18.7	19.4	62,200	91	poor	2	4	2 mos.	1.37
	Serum	6.69	32.3	10.8	17.3	17.6	22.0							
330748	Fluid	4.41	47.2	6.3	8.2	11.2	27.1	12,600	78	fair	3	3	4 yrs.	1.36
	Serum	7.44	43.1	6.7	12.0	11.3	26.9							
565573	Fluid	4.00	57.4	5.6	10.0	14.9	12.1	450	68	good	1	2	2 wks.	0.44
	Serum	6.06	53.1	5.5	12.6	15.7	13.1							
507225	Fluid	5.19	53.6	5.0	15.5	14.6	11.3	10,150	4	fair	1	2	2 wks.	1.09
	Serum	7.56	50.4	5.0	11.8	19.7	13.1							
								Group B						
147485	Fluid	5.45	51.3	5.4	9.4	9.8	24.1	11,300	36	fair	2	2	3 yrs.	0.72
	Serum	7.88	51.2	5.6	12.2	12.7	18.3							
389209	Fluid	5.94	32.3	6.6	8.1	17.1	35.9	25,900	94	fair	2	3	14 mos.	1.52
	Serum	7.87	32.5	7.4	12.2	13.5	34.4							
499948	Fluid	6.43	31.9	6.1	13.0	12.5	36.5	13,850	88	very poor	2	3	3 yrs.	1.22
	Serum	8.14	32.7	7.6	18.9	12.2	28.6							
636487	Fluid	5.09	48.0	7.1	10.6	14.1	20.2	14,200	74	very poor	3	2	1 yr.	1.38
	Serum	6.81	47.0	6.4	12.3	16.1	18.2							
								Group C						
350995	Fluid	5.63	42.2	4.1	7.5	11.0	35.2	7,900	69	very poor	3	4	9 yrs.	1.06
	Serum	7.32	46.0	5.7	10.9	14.8	22.6							
389847	Fluid	5.52	44.2	2.9	4.8	6.9	41.2	10,350	4	good	1	1	14 mos.	0.14
	Serum	7.25	59.8	3.7	9.0	12.6	14.9							

* Protein concentrations: 1.8 per cent; in sodium diethylbarbiturate buffer of pH 8.6 and 0.1 ionic strength.
† See Footnote 7 to text.

a peak migrating with a mobility intermediate to those of hyaluronic acid and albumin, similar to those described by Schürch, Viollier, and Süllmann (4). Similar findings have been reported in the literature by Chargaff, Ziff, and Moore (14) for the electrophoresis of mixtures of heparin and plasma and by Partridge (15) in his work on chondromucoid. These investigators assumed that this intermediate peak may represent a complex of polysaccharide and protein, but, as yet, there is not adequate evidence to support such an explanation for the finding made in this laboratory.

In two patients bilateral knee effusions were present, and fluid was aspirated for electrophoretic analysis. As shown in Table III, the distribution of protein components was the same in both knees. This is of interest in view of the finding that in many patients with bilateral effusions, the cytology, nature of mucin and total concentration of protein are quite similar in both fluids (6).

DISCUSSION

The results of the electrophoretic study of synovial fluids in rheumatoid arthritis show a wide range of distribution of the relative concentration of the individual protein fractions. The only relation between the electrophoretic pattern and the severity of the joint disease is that mild cases tend

to show patterns similar to those found in traumatic arthritis. A better correlation was found between the distribution of the protein fractions and the duration of the effusion. The protein patterns of fluids of short duration resembled those of traumatic fluids, while in those of longer duration the distribution of the individual proteins in the fluid approached that of the serum. Moreover, the relative concentration of γ-globulin tended to rise with persistence of the effusion.

The relationship of the synovial fluid proteins to those of serum is unknown. The fact that migration patterns of joint fluid, as demonstrated with the aid of Figures 1, 2 and 3, show electrophoretically well-defined proteins with mobilities similar to those of albumin, α_1-, α_2-, β- and γ-globulin of serum, does not prove chemical identity. The low concentration of albumin and globulin found in normal synovial fluid has generally been ascribed to slight capillary permeability (see review [16]) and the increase in joint effusions is assumed to be due to an alteration in this permeability. The relatively high concentration of albumin in traumatic and in many mild rheumatoid fluids suggests that the normal differential permeability to various protein fractions persists when the inflammation is mild. On the other hand, the similarity of the relative concentration of the individual fractions in serum and fluid in many cases of active, moderately severe rheumatoid arthritis suggests complete permeability in these cases.

In addition to the factor of permeability in determining the concentration of protein and the ratio of one fraction to another in the joint fluid, diminished rate of removal by the lymphatics and possibly production of one or more protein fractions locally in the tissues, either by a synthetic mechanism or by tissue destruction, may play a role. There is, as yet, no proof of the latter mechanism, but suggestive evidence that it is possible is furnished by one case of rather mild rheumatoid arthritis in which the serum protein pattern was entirely normal but the fluid contained 41.2 per cent of γ-globulin (Figure 3 and Table II, Case 389847).

SUMMARY

The electrophoretic patterns of 31 synovial fluids from 21 patients with rheumatoid arthritis have been determined and compared with those of traumatic effusions (representing relatively normal fluids).

The fluids could be divided into three groups: A) With a relative albumin concentration higher in the fluid than the serum; B) with albumin concentrations equal; and C) with the albumin fraction lower in the fluid.

The distribution of proteins suggests only a possible correlation with the severity of the disease, but does show a definite relation to the duration of effusion.

Fluids obtained from bilateral knee effusions in two patients gave identical electrophoretic patterns.

ADDENDUM

Electrophoretic Distribution of Proteins in Serum and Synovial Fluid in Other Rheumatic Diseases

Few synovial fluids from other joint diseases have been studied electrophoretically. However,

TABLE III

Electrophoretic distribution of proteins in serum and synovial fluid from bilateral effusions in rheumatoid arthritis *

Unit No.		Total protein *Gm./100 cc.*	Concentrations in per cent as					Joint fluid			Stage of disease†	Functional classification†	Duration of effusion *yrs.*	Sedimentation rate *mm./min.*
			Albumin	Globulins				Leukocytes *per cu. mm.*	Polymorphonuclears %	Type of mucin ppt.				
				α_1	α_2	β	γ							
210563	Fluid—L	5.26	47.7	3.2	5.5	7.7	35.9	4,750	8	good	1	1	5½	0.78
	Fluid—R	5.38	46.7	4.2	5.4	7.6	36.1	5,500	8	good			5½	
	Serum	7.06	48.5	4.7	8.5	13.3	25.0							
270773	Fluid—L	7.37	20.4	3.4	4.4	5.0	66.8	3,500	7	good	2	2	8	0.78
	Fluid—R	7.37	20.9	3.6	4.6	4.9	66.0	2,800	17	good			8	
	Serum	7.93	33.7	6.8	13.8	13.4	32.3							

* Protein concentrations: 1.8 per cent; in sodium diethylbarbiturate buffer of pH 8.6 and 0.1 ionic strength.
† See Footnote 7 to text.

TABLE IV

Electrophoretic distribution of proteins in serum and synovial fluid from various rheumatic diseases *

Unit No.		Total protein Gm./100 cc.	Concentrations in per cent as					Joint fluid			Sedimentation rate mm./min.
			Albumin	Globulins α_1	α_2	β	γ	Leukocytes per cu. mm.	Polymorphonuclears %	Type of mucin ppt.	
				Rheumatic Fever							
334510	Fluid	5.15	36.7	6.9	8.8	15.0	32.6	18,650	84	fair	1.71
	Serum	7.91	32.5	11.5	16.2	14.1	25.7				
291855	Fluid	4.58	43.3	9.1	12.9	15.9	18.8	8,500	73	fair	—
	Serum	7.56	36.4	9.3	17.7	14.9	21.7				
				Gout							
704258	Fluid	4.42	44.5	8.4	11.3	17.3	18.5	11,100	92	fair	—
	Serum	6.38	39.4	8.6	12.6	16.6	22.8				
				Reiter's Syndrome							
694675	Fluid	4.29	51.8	8.1	12.9	15.7	11.5	44,300	89	poor	0.97
	Serum	6.50	48.8	10.0	14.4	13.2	13.6				
705909	Fluid	5.36	51.1	6.5	12.6	16.6	13.2	2,566	22	very poor	1.70
	Serum	7.25	49.4	6.7	14.6	14.0	15.3				
				Intermittent Hydrarthrosis							
563722	Fluid	4.89	51.6	6.8	9.4	17.8	14.4	41,200	86	very poor	0.46
	Serum	6.94	47.7	6.2	12.7	18.2	15.2				
				Hypertrophic Pulmonary Osteoarthropathy							
694222	Fluid	3.27	45.1	13.6	12.5	13.1	15.7	3,000	44	fair	1.57
	Serum	5.87	33.6	12.4	21.2	15.3	17.5				

* Protein concentrations: 1.8 per cent; in sodium diethylbarbiturate buffer of pH 8.6 and 0.1 ionic strength.

comparison of the results available with those from rheumatoid and traumatic fluids is of interest.

The electrophoretic distribution of proteins in two synovial fluids from patients with rheumatic fever resembled that found in Group A of the rheumatoid fluids with the albumin concentration higher in the fluid than in the serum (Table IV). Two of the cases reported by Svartz and Olhagen (3) showed similar patterns, whereas in the third the concentrations in the fluid were essentially the same as in the serum.

In one patient with gout, two patients with Reiter's syndrome, one with intermittent hydrarthrosis, and one with hypertrophic pulmonary osteoarthropathy, the electrophoretic distribution of proteins in the synovial fluid resembled that in Group A of the rheumatoid fluids, the albumin content being higher in the fluid than in the serum (Table IV).

In all cases, as in the rheumatoid fluids, the α_1- and β-globulin concentrations were essentially the same in fluid and serum, and the α_2-globulin fraction was lower in the fluid.

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THE RENAL EXCRETION OF INOSITOL IN NORMAL AND DIABETIC HUMAN BEINGS [1]

By WILLIAM H. DAUGHADAY AND J. LARNER [2] WITH THE TECHNICAL ASSISTANCE OF ELLABETH HOUGHTON

(From the Departments of Medicine and Biological Chemistry, Washington University School of Medicine, and Barnes and Homer Phillips Hospitals, St. Louis, Mo.)

(Submitted for publication July 2, 1953; accepted October 28, 1953)

The presence of increased amounts of inositol in the urine of patients with diabetes mellitus has been known for almost a century (1–3). The inosituria in diabetes mellitus has generally been attributed to polyuria because the excretion of inositol has also been reported to be high in patients with diabetes insipidus. Some experimental support for this hypothesis has been provided by Needham (4), who observed a moderate increase in the excretion of inositol by rats made polyuric by feeding salt while on a low inositol diet.

Understanding of the metabolism of inositol has been delayed in part by the lack of specific and sensitive analytical methods. A new analytical procedure was made possible by the observation of Eastcott (5) that certain strains of yeast require inositol for optimal growth. The resultant microbiological assay has permitted the estimation of microgram quantities of inositol with satisfactory accuracy. In this report the inosituria of diabetes is confirmed using the yeast microbiological assay, and observations in man relating this abnormality to glucosuria rather than polyuria are presented.

METHODS

Inositol in urine

Inositol was measured by the method of Atkin, Williams, Schultz, and Frey (6). Unknown solutions were tested in duplicate at three dosage levels. The turbidity produced by the growth of Saccharomyces carlsbergensis in the tubes containing the unknown solution was compared to that developed in tubes containing known amounts of inositol and incubated simultaneously. Duplicate tubes containing seven different concentrations of inositol were prepared for each assay. Treatment with Deeminite [3] was introduced for the studies presented in Tables III and IV to remove pigments, inhibitory substances [4] and virtually all salts. The procedure used for free inositol was as follows: A dilution of urine was made so that the inositol content was between 2 and 8 micrograms per milliliter. To 50 ml. of such diluted urine was added 7 Gm. of Deeminite. After shaking intermittently for 30 minutes the slurry was filtered and the filtrate was used directly for assay.

Glucose added to normal urine was without significant effect on inositol measurements (Table I). Representative recoveries from a normal urine of relatively high and a urine of low inositol content are given in Table II. The very low concentration of inositol in certain urines presented special problems of assay. Maximum values were occasionally noted in the tubes containing the least amount of test solution suggesting the presence of inhibitors. Purification of the inositol in such a urine has been carried out to determine whether inhibitors present in the urine had masked large amounts of inositol. Saturated lead acetate was added to diluted urine at a pH of about 6. The resultant precipitate was discarded. The pH of the supernatant solution was then adjusted to about 10 with concentrated ammonium hydroxide. After standing over night in an ice box the precipitate was collected and washed with absolute alcohol. Inositol was eluted from the precipitate with water acidified to pH 3 with sulfuric acid. The resulting eluate was desalted with Deeminite and assayed for inositol. No evidence of unrecognized inositol was obtained and the purified solutions did not show inhibitory effects in the assay. Ninety-two per cent of added inositol was recovered from urine by this procedure (Table II).

The inositol of human urine exists almost exclusively in the free form as determined by assay before and after prolonged hydrolysis according to the method of Woolley (8). Loss of some inositol occurs under such drastic conditions and only from 50 to 93 per cent of added inositol was recovered. For these reasons most samples were assayed without hydrolysis.

Inositol in plasma

Total plasma inositol was measured by refluxing 4 ml. of plasma with an equal volume of concentrated hydro-

[1] This work was supported by a grant-in-aid from the Nutrition Research Foundation.

[2] Fellow of the Life Insurance Medical Research Foundation: Present address, Division of Bio-Chemistry, University of Illinois, Urbana.

[3] Obtained from A. S. Aloe and used without purification.

[4] Choline has been shown to inhibit the microbiologic assay for inositol (7, 8). Because choline is present in only trace amounts in urine and Deeminite removes 99 per cent of choline added to distilled water, choline is not considered a significant inhibitor in these assays.

TABLE I

Effect of urine glucose on inositol assay *

Urine sample ml.	Glucose added mg.	per cent	Inositol found µg./sample	mg./day
5	0	0	190	103
5	50	1	198	108
5	150	3	179	93
5	300	6	169	91

* The indicated amounts of glucose were added to 5 ml. of urine A. After diluting to 50 ml. with water the sample was desalted with Deeminite and analyzed for inositol.

chloric acid for 12 to 14 hours on a sand bath. The digest was evaporated to dryness at 100° C. under reduced pressure with a stream of air. The residue was taken up in 20 ml. of water and was decolorized with Deeminite. Using the above method 75 per cent of inositol added to plasma was recovered.

Free inositol was determined in plasma by dialyzing 4 ml. of plasma in a cellophane bag against 25 ml. of distilled water at 8° overnight. Aliquots of the dialysate were analyzed for inositol. Calculations were made on the assumption that the concentration after dialysis was equal inside and outside the bag. Eighty per cent of the inositol added to plasma could be recovered by this method.

Clearance studies

The renal clearance of endogenous creatinine was used as an index of glomerular filtration. Creatinine was measured by the method of Folin and Wu (9). Glomerular filtration determined in this manner was adequate for the purposes of this study. The inositol clearance was calculated from the free (dialyzable) plasma inositol and the free urine inositol values. Fifteen-minute urine collection periods were used and a sample of heparinized blood was obtained five minutes after the start of each collection period. One minute before the end of each period, 20 ml. of distilled water and 20 ml. of air were injected through the catheter. The contents of the bladder were expressed by suprapubic pressure.

General technics

The subjects for these studies were healthy medical students and patients on the wards of the Barnes Hospital and the Homer G. Phillips Hospital. Normal subjects were allowed to eat unrestricted diets and the diabetic patients ate standard diabetic diets. Strict dietary control of inositol intake was not attempted because information concerning the inositol content and availability of inositol in common foods is not at hand. A good mixed diet has been estimated to contain about 1 Gm. of inositol per day (10). Analysis of diabetic diets in this laboratory gave values between 300 and 900 mg. per day.

Toluene was used as a preservative for urine during collection. Aliquots of urine were refrigerated until assay. Urine sugar was measured by the method of Somogyi (11).

RESULTS

Inositol excretion in normal subjects

The daily inositol excretion in the urine by 11 non-diabetic subjects was measured. The results of 21 analyses gave a mean excretion of 37 mg. per day with a range of 8 to 144 mg. per day (Figure 1). Because of the frequent assertion that urine inositol is related to urine volume, the excretion of inositol has been plotted against the urine volume in Figure 2. The effect of oliguria produced by water restriction and polyuria by excessive hydration has been studied in two normal subjects. A

TABLE II

Recovery of inositol added to urine

Urine	Aliquot ml.	Inositol added µg.	Preparation of sample	Inositol µg. aliquot	Found mg./day
A	2	0	Standard	72	98
	2	100	Standard	158	—
				86% Recovery	
B	5	0	Standard	23(?)	13(?)
	5	100	Standard	138	—
				115% Recovery	
	10	0	Pb Precipitation*	<5†	—
	10	200	Pb Precipitation*	189	
				92% Recovery	
	50	0	Pb Precipitation*	80	3.9

* Purified by basic lead precipitation method described under *Methods*.
† Inositol content too low for accurate assay.

TABLE III

The plasma level and urinary excretion of inositol after the oral ingestion of 3 grams of inositol—normal and diabetic subjects

	Plasma inositol, *mg./100 ml.*				Urine inositol, *mg./day*			Urine glucose, *Gm./day*		
	0*	2 hrs.*	4 hrs.*	24 hrs.*	Day 1	Day 2†	Day 3	Day 1	Day 2†	Day 3
Normal subjects										
1	0.8	—	0.9	0.9	37	50	35	—	—	—
2	0.8	—	1.9	1.0	69	181	81	—	—	—
3	1.2	—	1.6	0.8	103	127	96	—	—	—
4	1.1	—	1.7	0.9	101	146	86	—	—	—
5	1.2	—	2.5	1.6	—	—	—	—	—	—
Mean	1.02	—	1.72	1.02	78	101	75	—	—	—
Diabetic subjects										
1	0.3	1.3	2.2	1.1	305	660‡	471	>41	63	131
2	0.8	1.4	1.9	1.2	304	563	—	82	43	40
3	0.7	3.8	3.3	0.9	698	1299	533	135	147	102
Mean	0.60	2.16	2.47	1.07	436	841	502	86	84	91

* Hours after the ingestion of 3 Gm. of inositol.
† Inositol administered on Day 2.
‡ The urine collected during the first four hours after inositol administration was lost.

TABLE IV

The renal clearance of inositol

	Normal		Diabetic		Normal and glucose		
Patient	LK	DM	ES	SW	MJ	BB	LR
Age	20	25	35	45	48	18	25
Surface area, M^2	1.74	1.40		1.69	1.82	1.48	1.52
Control period I—15 minutes							
Inositol, plasma, *mg. %*	0.62	0.52	0.56	0.58	0.85	0.31	0.48
Inositol, urine, *mg.*	0.09	0.12	17.2	10.2	0.21	0.09	0.10
Glucose, blood, *mg. %*	—	—	517	389	75	79	95
Glucose, urine, *Gm.*	—	—	9.5	2.6	.005	.003	.010
Clearance—Creatinine, *ml./min.*	96	78	165*	113	92	57	93
Clearance—Inositol, *ml./min.*	0.9	1.5	205*	117	1.7	1.9	1.4
Control period II—15 minutes							
Inositol, plasma, *mg. %*					0.95	0.32	0.52
Inositol, urine, *mg.*					0.32	0.15	0.10
Clearance—Creatinine, *ml./min.*					88	79	103
Clearance—Inositol, *ml./min.*					2.3	3.3	1.3
Loading period I—15 minutes			*Inositol loading*			*Glucose loading*	
Inositol, plasma, *mg. %*	56	75	57	58	1.2	0.44	0.53
Inositol, urine, *mg.*	764	697	1452	1215	9.6	5.3	5.5
Glucose, blood, *mg. %*	—	—	497	375	393	568	782
Glucose, urine, *Gm.*	—	—	5.8	2.1	3.6	2.8	6.8
Clearance—Creatinine, *ml./min.*	76	65	71	105	85	72	93
Clearance—Inositol, *ml./min.*	91	62	170	139	53	80	69
Loading period II—15 minutes			*Inositol loading*			*Glucose loading*	
Inositol, plasma, *mg. %*	51	70	53	48	1.4	0.54	0.66
Inositol, urine, *mg.*	852	621	884	716	9.0	4.2	9.6
Glucose, blood, *mg. %*	—	—	506	355	334	550	588
Glucose, urine, *Gm.*	—	—	4.1	1.6	2.5	2.5	6.2
Clearance—Creatinine, *ml./min.*	135	59	60	97	78	74	104
Clearance—Inositol, *ml./min.*	112	60	111	100	43	52	97

* The high clearances in this period can be attributed to faulty urine collection. The ratio of inositol to creatinine clearance should be unaffected.

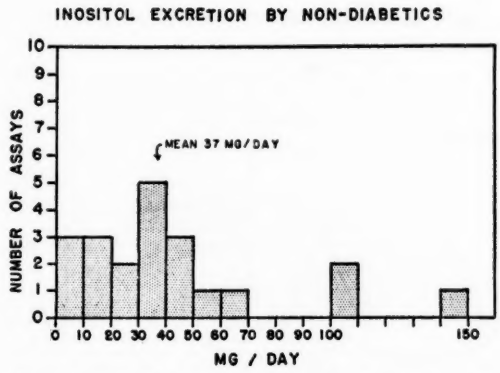

FIG. 1. THE URINARY EXCRETION OF INOSITOL BY 11 NON-DIABETIC SUBJECTS ON RESTRICTED DIETS

three-fold increase in urine volume produced only an insignificant increase in urine inositol. Much more inositol was found in the urine of diabetic subjects with glycosuria in excess of 75 Gm. a day than in the urine of non-diabetic subjects at comparable urine volumes (Figure 2).

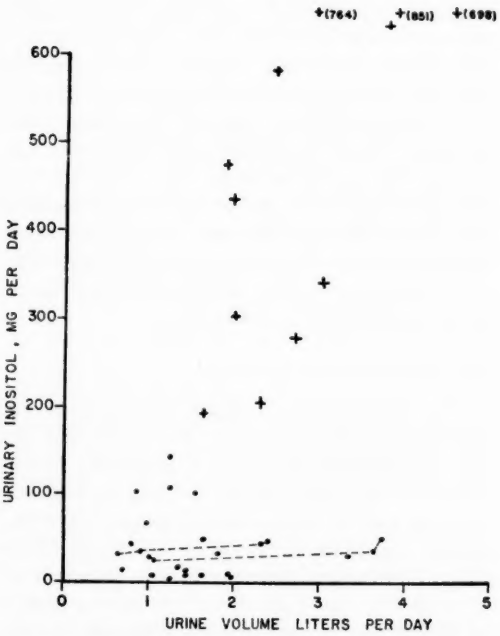

FIG. 2. THE DAILY EXCRETION OF INOSITOL BY NON-DIABETIC (●) AND DIABETIC (+) SUBJECTS PLOTTED AGAINST URINE VOLUME

The broken lines connect values from the same individual during water restriction and over-hydration.

Inositol excretion in diabetes

Repeated observations have been made of the daily inositol excretion of seven hospitalized patients with uncontrolled diabetes mellitus. From 280 to 851 mg. per day of inositol were excreted during severe glycosuria. One representative subject (Figure 3) was given decreasing doses of insulin until the urinary excretion of glucose increased to more than 100 Gm. per day. The inositol in the urine was measured both with and without hydrolysis and the results agreed within the accuracy of the method. During the period of uncontrolled diabetes, large amounts of inositol were present in the urine. Upon reinstitution of insulin treatment, the excretion of inositol fell to the normal range.

A second representative case, (Figure 4) exhibited severe glycosuria during a period of insulin resistance. Before insulin therapy became effective between 479 and 851 mg. of inositol were excreted daily. With improved diabetic control, the inositol excretion fell. The urine volume in both of these cases did not exceed 3.8 liters despite glycosuria in excess of 100 Gm. per day.

Inositol load experiments

The effect of the oral ingestion of inositol on plasma concentration and urinary excretion has been studied. After a control day, a single dose of 3

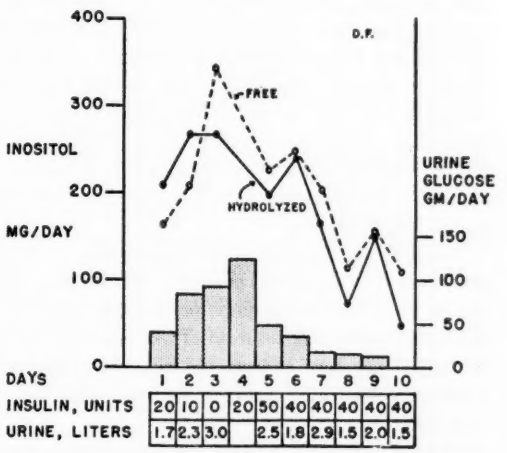
D.F.
FREE
HYDROLYZED
INOSITOL MG/DAY
URINE GLUCOSE GM/DAY
DAYS 1 2 3 4 5 6 7 8 9 10
INSULIN, UNITS 20 10 0 20 50 40 40 40 40 40
URINE, LITERS 1.7 2.3 3.0 2.5 1.8 2.9 1.5 2.0 1.5

FIG. 3. THE DAILY EXCRETION OF FREE AND HYDROLYZABLE INOSITOL BY A REPRESENTATIVE PATIENT WITH UNCONTROLLED DIABETES

The excretion of glucose is shown in the shaded columns.

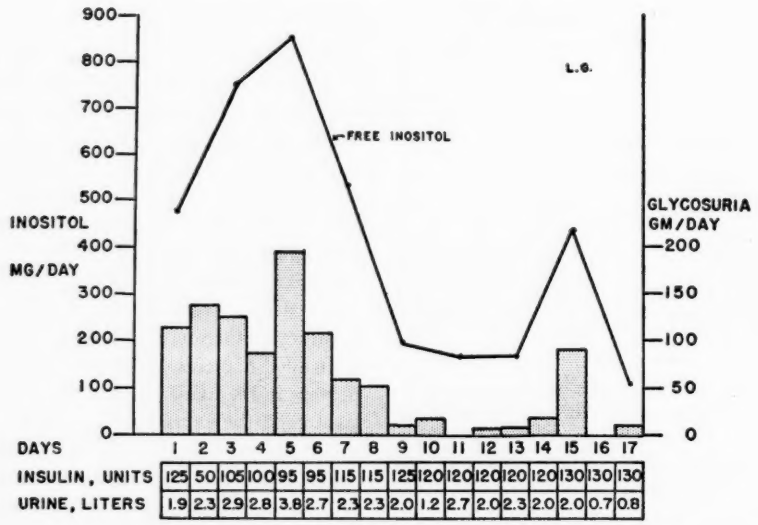

FIG. 4. THE DAILY EXCRETION OF INOSITOL BY A SECOND REPRESENTATIVE PATIENT WITH UNCONTROLLED DIABETES

The excretion of glucose is shown in the shaded columns.

Gm. of inositol dissolved in 200 ml. of tap water was given by mouth on the morning of the second day. This dose of inositol was estimated to be from three to five times the normal dietary intake. Breakfast was omitted, but, thereafter, the subjects were allowed to eat their regular diet. The inositol excretion was measured on the control day and on the two days after inositol ingestion. The inositol excretion by normal subjects, Table III, increased 23 mg. per day following inositol ingestion. The increase in inositol excretion by the diabetic patients averaged 405 mg. per day. The plasma levels of inositol of diabetic subjects before inositol loading were within the same range as those of the non-diabetic. The plasma inositol rose in both normal and diabetic subjects.

Inositol has been given intravenously to three control and five diabetic patients. Glycosuria was severe in all but one diabetic patient who only had mild glycosuria. A solution of 0.2 per cent inositol in 0.9 per cent sodium chloride was administered in a dose of 20 mg. per Kg. over a period of four hours.

The plasma inositol levels following intravenous administration of inositol were essentially similar when measured either by the hydrolysis or dialysis techniques. Only the results obtained by the latter method have been used in plotting Figure 5. The plasma inositol level rose 200 to 400 per cent in both diabetic and control subjects during the infusion and promptly fell after the end of the infusion.

The diabetic patients excreted much more inositol than the control subjects on the preinfusion day. The inositol increment on the day of inositol infusion amounted to 36 per cent of the administered dose in the diabetic and only 13 per cent in the normal subjects. On the day after inositol infusion, the excretion in the urine had returned nearly to the control level in both groups.

The renal clearance of inositol

The clearance of endogenous creatinine and inositol have been measured in two diabetic and two non-diabetic patients. Endogenous inositol clearance was very low in the two normal subjects and high in the two diabetic subjects, approximately equivalent to creatinine clearance (Table IV). The inositol clearance at high plasma levels of inositol has been measured. Seventy milliliters of 10 per cent inositol was given rapidly by vein. The loading dose was followed by the injection of a sustaining solution containing one per cent inositol in isotonic saline given at a rate of 4 ml. per minute. After a five-minute equilibration period inositol

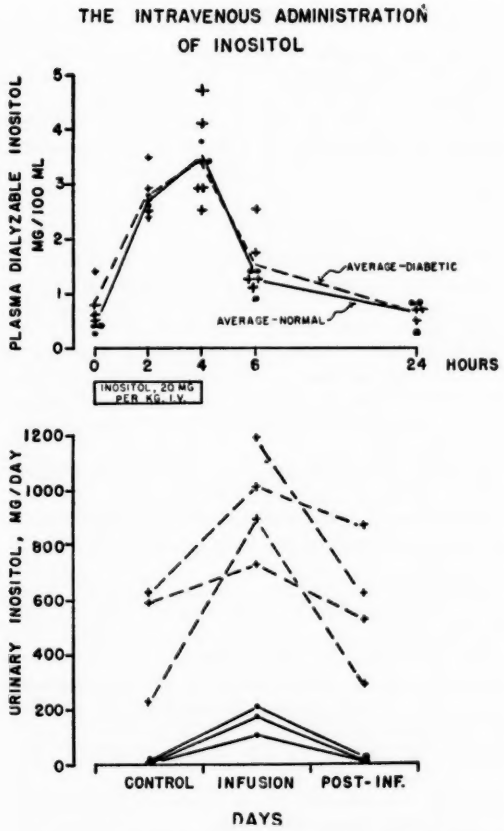

FIG. 5. CHANGES IN PLASMA DIALYZABLE INOSITOL AND URINE INOSITOL EXCRETION BEFORE AND AFTER THE INTRAVENOUS ADMINISTRATION OF 20 MG. PER KG. BODY WEIGHT OF INOSITOL FOR FOUR HOURS

Values obtained from diabetic patients are indicated by (+), those for normal patients by (●).

and creatinine clearances were determined. With plasma inositol levels between 51 and 75 mg. per 100 ml. the clearance of inositol rose to levels comparable to creatinine clearance in both normal and diabetic subject.

The effect of glucose loading on inositol excretion has been studied in three non-diabetic subjects. One half-hour prior to the clearance measurements the patients received 3 Gm. of inositol by mouth. The inositol clearance of these subjects was low during the control period (Table IV). Glucose loading was performed by the rapid intravenous administration of 50 per cent glucose in a dose of 2 ml. per Kg. of body weight for M. J. and 2½ ml. per Kg. of body weight for the other two subjects. When the priming dose of glucose had been given, a sustaining solution of 20 per cent glucose was started at a rate of 4 ml. per min. and was continued for two 15-minute clearance periods. A sustained hyperglycemia and glycosuria were achieved in these patients. A 30-fold increase in inositol clearance occurred during glucose infusion.

DISCUSSION

The present observations confirm earlier reports of an increased urinary excretion of inositol in human diabetes mellitus. Since urine volume correlated poorly with inositol excretion in non-diabetic subjects, other causes of the inosituria have been sought.

These studies indicate that an alteration of renal function is responsible for the inosituria of diabetes. Filtered inositol is almost completely reabsorbed at physiologic plasma levels in normal subjects. With plasma levels of inositol between 51 and 75 mg. per 100 ml., the ability of the tubule to reabsorb inositol is exceeded, and inositol clearance approaches the rate of filtration. A striking difference exists in patients with diabetes mellitus. Large amounts of inositol are excreted even at normal levels of plasma inositol. The renal clearance of inositol in non-diabetic subjects rose greatly when a massive load of glucose was presented to the renal tubules. It would seem clear that glucose increases the excretion of inositol probably by inhibiting the reabsorption of inositol by the human renal tubule.

A comparable situation exists in the reabsorption of xylose by the renal tubule. Glucose and phlorizin have been found to block the reabsorption of xylose suggesting that glucose and xylose may share the same transport mechanism (12). Phlorizin also inhibits the reabsorption of inositol by the renal tubules in rats (13). At low plasma levels the reabsorption of inositol by the renal tubules is much more complete than the reabsorption of xylose. As the plasma level is raised the renal clearance of both substances approaches the rate of glomerular filtration. Further study is necessary to establish the relation between inositol and glucose transport mechanisms.

SUMMARY

1. The urine of 11 non-diabetic subjects contained an average of 37 mg. of inositol per day (range 8 to 144 mg.). Urine volume correlated poorly with inositol excretion.

2. Seven uncontrolled diabetic patients had a urinary excretion of from 280 to 851 mg. per day of inositol. The inosituria of diabetes disappeared after the control of glycosuria.

3. A small rise in the plasma level of inositol occurred following the ingestion of 3 Gm. of inositol in both diabetic and non-diabetic subjects. The average increase in urinary excretion on the day of inositol ingestion was 23 mg. in the control group and 405 mg. in the diabetic group.

4. Diabetic subjects excreted three times more inositol in their urine than did non-diabetic subjects following the slow intravenous administration of 20 mg. per Kg. of inositol but the levels of plasma dialyzable inositol were the same in the two groups.

5. The renal clearance of inositol in normal subjects at physiologic plasma levels of inositol was low. A 3000 per cent increase in inositol clearance occurred following glucose loading at normal plasma inositol levels. At high plasma inositol levels clearance rose to the level of endogenous creatinine clearance. The renal clearance of inositol in diabetic subjects was high even at normal plasma inositol levels.

6. It is concluded that a renal tubular mechanism for the reabsorption of inositol exists and that tubular transport of inositol is inhibited by high glucose loads. The inosituria of diabetes mellitus can be attributed to an increased inositol clearance produced by glycosuria and not by polyuria.

ACKNOWLEDGMENTS

The authors wish to acknowledge the assistance of Mrs. M. Heady, laboratory technician, and Miss S. Wood, research nurse, in the performance of this study.

The pyrogen-free inositol for parenteral use was generously provided by Dr. H. J. Byrne of the Commercial Solvents Corporation, Terre Haute, Indiana.

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THE BIOSYNTHESIS OF SQUALENE FROM ACETATE IN MAN[1]

By MAXWELL L. EIDINOFF, ROBERT S. ROSENFELD, JOSEPH E. KNOLL, BENJAMIN J. MARANO, AND LEON HELLMAN

(From the Divisions of Physics and Biophysics and of Steroid Biochemistry, Sloan-Kettering Institute for Cancer Research, New York, 21, N. Y.)

(Submitted for publication August 3, 1953; accepted October 21, 1953)

INTRODUCTION

The recently reported experiments of Langdon and Bloch (1, 2, 3) have furnished significant support to the hypothesis that the unsaturated hydrocarbon squalene ($C_{30}H_{50}$) is an intermediate in the pathway from acetate to cholesterol. These experiments were carried out in intact rats, as well as in rat tissue slices. Our laboratory has undertaken an investigation of the role of this hydrocarbon in metabolic pathways in the human. It has been established that the liver is the major source of plasma cholesterol (4). However, Dimter (5) and Stanger, Steiner, and Bolyard (6) were not able to isolate or identify squalene in the livers of the human and several other mammalian species. If the intermediate role of squalene is accepted, it would necessarily follow that the squalene "pool" in the liver is of very small size but turns over extremely rapidly.

The experiment reported here was designed to ascertain whether acetate is a precursor for squalene biosynthesized by the human. It has already been demonstrated that acetate is an important carbon source for cholesterol (7, 8). Since small quantities of squalene are present in human sebum, the oily secretion of the skin (9), this material was chosen as the only accessible squalene source in this experiment. The specific activities of the squalene and total cholesterol in the sebum as well as that of plasma cholesterol were measured following oral administration of acetate-2-C^{14}. The results given below demonstrate that acetate is a precursor for squalene in the human and that the squalene in human sebum is not simply derived from exogenous substances or exclusively from their degradation products.

[1] This work was supported in part by contract AT(30-1)-910 with the United States Atomic Energy Commission and in part by a grant (C-440) from the National Cancer Institute, United States Public Health Service.

EXPERIMENTAL

Permission to administer acetate-2-C^{14} to a patient of limited life expectancy was obtained from the Atomic Energy Commission. The patient, a 32 year-old white female, had a functioning adrenal carcinoma. Three hundred microcuries were administered orally in a solution containing 50 mg. of sodium acetate.[2] Since experimental studies of this type in the human involve difficulties beyond those normally encountered in animal studies, data are here presented only for this one patient. As squalene is a normal component of human sebum, a demonstration in only one patient that squalene containing radiocarbon is synthesized in the human is a justifiable procedure. Sebum was collected by gently washing the skin on the arms, legs, back and scalp, using cotton swabs soaked with acetone. Precautions were taken to insure that salves or lotions, etc., were not applied to the parts used for sebum collection. There appeared to be no discomfort to the patient following this procedure.

The chloroform extraction procedure of MacKenna, Wheatley, and Wormall (10) was used after the acetone had been removed by distillation under reduced pressure in an atmosphere of nitrogen. Following the removal of the chloroform by distillation, the residue was saponified by standard procedures using aqueous-alcoholic potassium hydroxide solution. The non-saponifiable fraction was extracted with ether and, after the removal of the solvent, was then dissolved in 50 ml. of petroleum ether and poured onto a 1 cm. diameter column containing a quantity of Merck acid washed alumina 50 times the weight of the non-saponifiable portion. The hydrocarbon fractions were eluted with 250 ml. of petroleum ether (60°) and subsequent elutions with 10, 20, 30, 40, and 50 volume per cent benzene in petroleum ether were used in order to obtain the sebum cholesterol fraction.

Preparation and analysis of squalene hexahydrochloride. After removal of the petroleum ether by distillation the hydrocarbon residue was mixed with a small volume of acetone (at least ½ ml. and generally 3 times the volume of non-saponifiable fraction). Substances not soluble in this volume of acetone at room temperature were removed by centrifugation. The hexahydrochloride derivative was then prepared by treatment with dry HCl at −5° for several hours. The white crystalline precipitate was washed repeatedly with absolute ether at 5°. The yields

[2] The labeled acetate was obtained from Tracerlab, Inc., after authorization from the Isotopes Division, U. S. Atomic Energy Commission.

TABLE I

Analytical data for squalene hexahydrochloride preparations*

Compound	Sample number	Remarks	Percentage by weight		
			Carbon	Hydrogen	Chlorine
Squalene hexahydrochloride	1H	Sebum-scalp	57.50	9.09	33.41
	2H	Sebum-scalp	57.22	8.78	33.59
	C	Isomer mixture	57.46	9.17	33.69
	A	113° M.P. isomer	57.42	8.93	33.58
	B	143° M.P. isomer	57.32	8.93	33.65
Calculated for $C_{30}H_{56}Cl_6$			57.23	8.97	33.79
Squalene		Redistilled	87.74	12.39	
Calculated for $C_{30}H_{50}$			87.73	12.27	

* These microanalyses were carried out by Mr. J. Alicino, Metuchen, N. J.

of $C_{30}H_{56}Cl_6$ from samples 1, 3, and 4 (Table II) were 3 to 5 mg., sufficient for radioactive assay, infra-red and x-ray powder pattern analysis. The sebum samples from the scalp yielded larger quantities of the halogen derivative. In the case of sample 1H, the weights of fat, non-saponifiable material, hydrocarbon fraction and $C_{30}H_{56}Cl_6$ were 3.1, 1.2, 0.23, and 0.027 Gm., respectively. In the case of 2H, 0.050 Gm. of $C_{30}H_{56}Cl_6$ was obtained from 2.3 Gm. of fat.

The elementary analyses for the squalene derivatives derived from the sebum or used as standards are listed in Table I. Samples A, B, and C were prepared from a redistilled sample of commercial technical grade squalene (Distillation Products Industries), obtained originally by molecular distillation of basking shark liver oil. The molecular distillation was controlled by using an iodine value as an index of purity of the squalene. The elementary analysis of the redistilled squalene agreed with the theoretical (Table I). In addition to elementary analysis, two methods were used to demonstrate that the halogen-containing derivative obtained from the hydrocarbon portion of the eluate was squalene hexahydrochloride. The infra-red spectrum of the samples derived from sebum in Table II were compared with that for standard sample C (Table I) using a double beam Perkin-Elmer spectrophotometer Model 21. Approximately 2 mg. of the $C_{30}H_{56}Cl_6$ samples from sebum (Table II), the standard sample (C) and the low melting isomeride (A-Table I) were shaken with 150 mg. of carbon disulfide. The resulting mixtures were incompletely soluble. All these mixtures (with the exception of sample 1-Table II) exhibited very similar absorption peaks, the most useful for comparison purposes at wave numbers of 1380, 1308, 1265, 1237, 1210, and 745. It was also observed that the high melting isomeride (B-Table I), mixed in the same proportion, did not furnish absorption peaks in this interval. This indicates a relatively low solubility of the 143° M.P. isomeride. The relative intensities of several peaks did not agree with the standard in the case of sample 1. The total amount of sample (about 3 mg.) did not permit any purification procedures. However, sample 1H, which covers a corresponding period of the experiment was available in sufficient quantity for precise comparison of elementary analysis, infra-red spectrum and x-ray diffraction powder photographs.

The x-ray diffraction powder photographs for the crystalline sebum squalene derivatives (Table II) were compared with standard sample C (Table I). No significant differences were detectable in all the photographs. This was also the case for the low melting derivative (Sample A-Table I). Samples A and B were prepared from C following the procedure outlined by Heilbron, Kamm, and Owens with modifications noted by Langdon and Bloch (2, 11). The powder pattern photographs were made by Dr. Benjamin Post in the X-ray Diffraction Laboratory of Brooklyn Polytechnic Institute, using a 57.32 mm. radius camera and filtered copper radiation.

Cholesterol analysis. The fractions eluted from the column with 30 to 50 per cent benzene in petroleum ether contained the sebum cholesterol. These fractions were combined and recrystallized twice from acetone yielding crystals melting at 144–46°. The cholesterol was precipitated as the digitonide for radiocarbon assay (12). The plasma cholesterol was isolated by a modification of the Sperry-Webb procedure (12).

Analysis for radiocarbon specific activity. All samples were assayed by solid plating of squalene hexahydrochloride and cholesterol digitonide in shallow stainless steel cups having a 1.63 $cm.^2$ area. Windowless gas-flow counters were used (Tracerlab Model SC-16). Correlation of observed counting rate and specific activity was made using intercalibration data obtained by the internal gas counting procedures described by Eidinoff (13, 14).

RESULTS AND DISCUSSION

The specific activities of the squalene and total cholesterol in five sebum samples are listed in Table II. The maximum squalene specific activity, 142 dpm per micromole, was found in sample 2H. The results in column 4 prove that the methyl carbon of acetate is a precursor for squalene synthesis and that sebum squalene is not simply de-

rived exclusively from exogenous sources or their degradation products.

The squalene specific activities were greater than those for the cholesterol in the sebum samples collected in the first 16 days. The plasma free cholesterol specific activities were considerably greater than those for either sebum squalene or cholesterol in the early part of the experiment and remained greater for the entire 24-day period of the experiment.

However, any attempts to compare these specific activities as functions of time in order to yield information on dynamic interrelationships meet with two serious difficulties. The first involves the collections of the sebum samples. It was not feasible to investigate the effect on the specific activities of variations in the manner, duration, and frequency of the acetone washings and of the sites used for sebum collection. The second difficulty is concerned with the physiological details of sebum formation and transport to the skin surface.

Srere, Chaikoff, Treitman, and Burstein showed that the skin and liver of the adult rat were the most active tissues with respect to the utilization of acetate for cholesterol formation *in vitro* (15). If this is assumed to be the case for the human, then the skin cholesterol specific activity may reach a maximum value within several hours after the administration of the labeled acetate, as does (Table II) plasma cholesterol (12) which is chiefly derived from cholesterol synthesized in the liver (4, 16). If it is further assumed that the squalene is an intermediate in cholesterol synthesis (2), then it is probable that both the skin squalene and cholesterol would also reach their maximum specific activities within several hours. However, the transport of a portion of these products as components of the sebum to the external surface of the skin may involve differential transfer and solubility effects. As a result, the squalene-cholesterol specific activity ratio in the sebum may not correspond to that prevailing in the interior layers of the skin at the time these materials were synthesized. Furthermore, unlabeled squalene and cholesterol present at the start of the experiment in the crypts of the glandular elements of the skin must be first removed before the newly labeled sebum is in a position to be collected. This is probably the reason for the low specific activity of the constituents in sebum collection 1 and 1H relative to the plasma free cholesterol activity in this interval. In addition to the *in situ* formation of cholesterol in the skin, cholesterol labeled with radiocarbon and synthesized in the liver is transported to the skin.

There is no clear explanation for the relatively high squalene content of sebum as compared to the as yet undetectable amounts found in human liver and spleen (5, 6, and our own unpublished experiments). Sebum contains debris of degenerated epithelial tissue. It is possible that the enzyme systems associated with cholesterogenesis are partially interrupted in those cutaneous cells which are in the process of being sloughed off into the sebum and that the squalene accumulates as a result of this metabolic alteration.

SUMMARY

1. The biosynthesis of squalene, a hydrocarbon which may be an intermediate in cholesterol synthesis, has been demonstrated in man by finding radiocarbon activity in sebum squalene after the administration of acetate-2-C^{14}.

TABLE II

Specific activity of squalene and total cholesterol in sebum samples following administration of acetate-2-C^{14}

Sample number	Source of sebum	Interval	Specific activity (*dpm/micromole*)		
			Sebum squalene	Sebum cholesterol	Plasma* free cholesterol
		(*days*)			
1	Arms, legs, back	0.3– 2.3	8 ± 1	4 ± 2	790
1H	Scalp	0– 2.4	9 ± 0.5	<1	790
2H	Scalp	2.4– 7.3	142 ± 2	98 ± 2	420
3	Arms, legs, back	9 –16	59 ± 2	34 ± 2	205
4	Arms, legs, back	19 –24	17 ± 1	72 ± 2	114

* At midpoint of sebum collection interval (column 3).

2. The specific activity of sebum squalene was compared with sebum and plasma cholesterol at several intervals over a period of 24 days and the implications of these findings are discussed.

ACKNOWLEDGMENT

The authors are indebted to Miss F. Herling of the Division of Steroid Metabolism for the infra-red analyses and to Dr. T. F. Gallagher for helpful discussions.

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OBSERVATIONS ON THE VISCERAL REMOVAL OF PLASMA PEPTIDASE ACTIVITY[1]

By THEODORE B. SCHWARTZ[2] AND J. D. MYERS

(From the Department of Medicire, Duke University School of Medicine, Durham, N. C.)

(Submitted for publication August 7, 1953; accepted November 4, 1953)

Fluctuation in activity of plasma peptidases has been noted as a concomitant of a wide variety of clinical disorders. These proteolytic enzymes exhibit enhanced activity in the plasma of man or experimental animals subjected to any severe tissue damage, and there is convincing evidence, discussed elsewhere (1, 2), that these enzymes gain entrance to the circulation from injured or destroyed cellular components. With healing (3) or clinical remission (4, 5), elevated plasma peptidase values return to the normal range, but the mechanisms involved in the removal of peptidase activity from the plasma have not been systematically investigated. The present study was undertaken in an attempt to determine the fate of these circulating peptidases.

MATERIALS AND METHODS

A. Nephrectomy in rats

After an 18-hour fast, male rats of the Vanderbilt strain weighing 200 to 250 Gm. were bilaterally nephrectomized using pentobarbital anesthesia and a lumbodorsal operative approach. Twenty-four hours later the animals were reanesthetized, and heparinized blood was collected from the abdominal aorta. Control blood samples were similarly obtained from rats not subjected to nephrectomy.

B. Studies of splanchnic removal of plasma peptidase in man

Accepted techniques were employed for collecting simultaneous femoral arterial and hepatic venous blood samples and determining hepatic blood flow, BSP clearance, net splanchnic oxygen consumption and net splanchnic glucose production (6–8). Peripheral venous blood was obtained from the antecubital vein.

The product of the estimated hepatic blood flow and the corresponding femoral arterial-hepatic venous difference of a metabolite gives the splanchnic balance of that material. Although it is probable that hepatic activity determines the balance in greatest part, it must be remembered that those viscera drained by the portal vein (gut, spleen, pancreas, etc.) play some role in the determination of splanchnic balance. Accordingly, the term splanchnic, meaning the domain of the liver plus the various viscera drained by the portal vein, is adhered to throughout this paper.

C. Determination of peptidase activity

In all instances, peptidase activity was measured by a photometric ninhydrin technique (9), using *non-hemolyzed* heparinized plasma. Two synthetic tripeptides, glycylglycylglycine (GGG) and L-leucylglycylglycine (LGG), were used as substrates, the former for studies on rats and the latter for measurement of splanchnic A–V differences.

Enzymic hydrolysis proceeded under conditions previously described (4, 9). Samples were withdrawn after one and two hours of incubation and the rate of hydrolysis taken as the mean of the two determinations, since the reaction, initially, appeared to follow zero order kinetics. The assumption that there is cleavage of only one peptide bond was fortified by the finding that, in a third sample, routinely taken eighteen hours after the initiation of incubation, the degree of hydrolysis calculated on this basis often equaled, but never exceeded, the 100 per cent value for one-bond cleavage. In order to quantitate changes in LGG-splitting in terms of hepatic blood flow, it was decided, somewhat reluctantly, to establish a *unit* of peptidase activity which is here defined as that *amount of enzyme activity in 1 ml. of plasma which hydrolyzes 1 μM of substrate per hour* under the conditions detailed above.

RESULTS

The kidney, as a site of removal of plasma peptidase, was investigated using bilaterally nephrectomized rats. Twenty-four hours after nephrectomy, in five animals, plasma GGG-cleaving activity was found to be 7.8 ± 1.1 units,[3] which differs insignificantly ($p < 0.2$) from the mean of 9.0 ± 0.1 units in twenty-nine normal animals. These findings, together with the report (1) of normal peptidase activity in patients in advanced uremia, make it seem quite unlikely that the kidney serves as an important avenue of excretion for these enzymes.

[1] This work was supported, in part, by grants from the National Institutes of Health, United States Public Health Service, the Duke University Research Council, and the Life Insurance Medical Research Fund.

[2] Damon Runyon Senior Clinical Research Fellow (1950–1952).

[3] Mean ± standard error.

TABLE I

Splanchnic uptake of plasma peptidase activity

Patient	Diagnosis	Procedure	Arterial plasma LGGase activity (*units*)	BSP clearance (*% of normal*)	Splanchnic plasma flow (*ml./min./sq. M*)	Splanchnic peptidase A-V difference (*units*)	Splanchnic LGGase uptake (*units per min. per sq. M*)	Net splanchnic oxygen consumption (*ml./min./sq. M*)	Net splanchnic glucose production (*mg./min. per sq. M*)
Group A—Normal Arterial Plasma LGGase Activity									
O. F.	Psychoneurosis	Control	5.46	87	390	−0.24	− 94	34	56
M. B.	Psychoneurosis Obesity	Cortisone 200 mg.	3.43	91	580	0.25	145	51	115
M. H.	Psychoneurosis	Cortisone 200 mg.	5.04	106	680	−0.43	−292	50	94
J. A.	Psychoneurosis	Cortisone 200 mg.	5.36	92	550	0.17	94	32	51
J. R.	Hysteria	Pressure study	4.26			−0.28			
A. G.	Cirrhosis	Pressure study	4.33			0.06			
	Mean ± S.E.		4.65± 0.319	94± 4.1	550± 60.1	−0.01± 0.109 0.4<p<0.5	− 37± 62.9 p>0.5	42± 5.1	79± 15.4
Group B—Elevated Arterial Plasma LGGase Activity									
J. S.	Peptic ulcer	Control	6.23	96	440	0.89	393	30	50
H. M.	Peptic ulcer	Oxypolygelatin infusion	7.86	128	790	1.46	1153	45	
J. H.	Optic neuritis	Typhoid vaccine	9.60	54	880	0.55	484	53	103
S. D.	Psychoneurosis	Cortisone 200 mg.	8.55	75	430	0.70	301	37	54
R. E.	Psychoneurosis	Cortisone 200 mg.	10.18	79	640	0.69	442	36	48
L. N.	Psychoneurosis	Cortisone 200 mg.	11.28	108	490	1.66	813	38	46
E. R.	Infectious hepatitis, convalescent	Pressure study	6.82			0.02			
G. W.	Infectious hepatitis	Pressure study	16.42			1.16			
W. F.	Homologous serum jaundice	Pressure study	17.99			0.91			
	Mean ± S.E.		10.55± 1.370	90± 10.7	612± 77.9	0.89± 0.164 p<0.01	598± 132 p<0.01	40± 3.3	60± 8.8

Attention was next directed to the role of the splanchnic area in the extraction of peptidases from the plasma. LGG-hydrolyzing activity was measured in simultaneously obtained samples of plasma from the femoral artery and hepatic vein of fifteen patients. In the eight instances in which replicate samples were collected, mean values were used. Since in these individuals hepatic vein catheterizations were performed primarily for other purposes, as indicated in Table I, the diverse nature of the procedures which were carried out in some instances undoubtedly influenced some of the measures of splanchnic circulatory and metabolic activity and conceivably could have provoked changes in peptidase levels as well. However, it was possible to obtain control blood samples in all but the cortisone studies (10), and there is now ample evidence (4) that plasma peptidase levels are not *directly* affected by changes in adrenal cortical activity. These facts may offer some reassurance regarding the validity of absolute values of plasma peptidase activity obtained; but, in actuality, there would appear to be little need to provide justification for the procedures used since the primary aim was to determine quantitatively whether the splanchnic area extracted peptidases from the plasma, regardless of any disease or experimental manipulation which might have produced changes in arterial peptidase levels.

Pertinent data are presented in Table I. The patients are divided into two groups, one consisting of those with normal arterial LGG-splitting activity (Group A) and the other of individuals with elevated plasma peptidase levels (Group B). This selection was based on the determination of plasma LGG-hydrolyzing activity in fourteen "normal" subjects. The mean value in this group was 4.47 ± 0.73 units,[4] so arterial plasma enzyme levels above 5.93 units (mean plus 2 S. D.) were considered to be increased beyond the normal range.

In twelve of the fifteen patients, arterial-hepatic venous peptidase differences were positive, suggesting that enzyme was being "extracted" from the plasma in the splanchnic area. Although it is apparent that in all of the patients these differences were of a rather low magnitude, a comparison of

[4] Mean ± standard deviation.

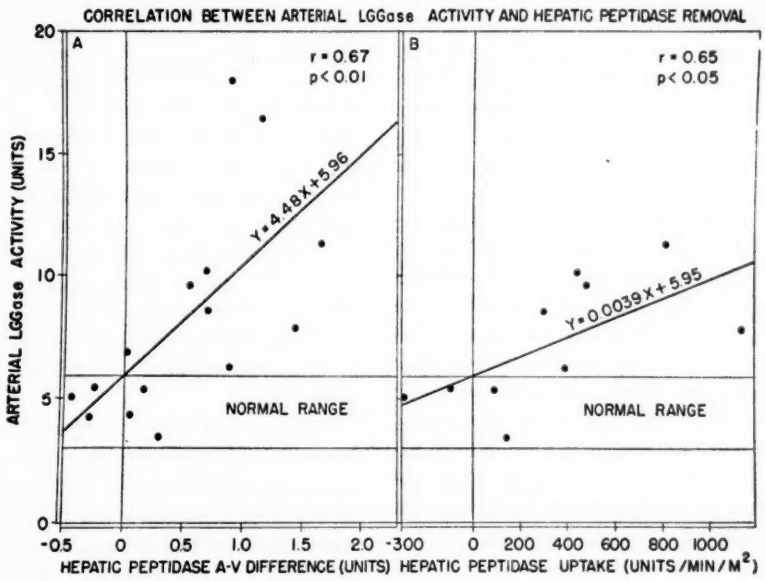

FIG. 1. THE STATISTICALLY SIGNIFICANT CORRELATION THAT EXISTS BETWEEN ARTERIAL PEPTIDASE ACTIVITY AND BOTH SPLANCHNIC PEPTIDASE ARTERIOVENOUS DIFFERENCE AND SPLANCHNIC UPTAKE IS SHOWN

The normal range of plasma LGG-splitting activity is defined by the mean value ± 2 standard deviations (4.47 ± 1.46) in fourteen normal subjects.

the two groups as indicated in Table I proved to be of interest. In Group A the hepatic A–V differences and the splanchnic uptakes were small or negative, so that mean values did not differ appreciably from zero ($p < 0.4$ and $p < 0.5$, respectively). In striking contrast, in Group B mean values for hepatic A–V differences and splanchnic uptakes were significantly increased above both zero and the appropriate mean values of Group A ($p < 0.01$ in all instances). Thus, it seems clear that in most patients with high plasma peptidase activity, significant quantities of enzymic activity are removed from the circulation by the liver and associated portal viscera, while no consistent change is detectable in normal plasma peptidase activity during passage through the splanchnic circulation.

Groups A and B are not distinguishable on the basis of differences in BSP clearance, hepatic plasma flow, net splanchnic oxygen consumption, or net splanchnic glucose production. The individual variations that occur are explicable in terms of the patient's illness or the procedure to which he was subjected.

The highest plasma peptidase levels in this series were found in patients G. W. and W. F., both ill with acute inflammatory liver disease. Indeed, the highest human plasma peptidase activity observed in this laboratory, 20.8 units, occurred in a patient in hepatic coma. This is in contrast to the slightly elevated value in E. R., who was convalescing from an attack of infectious hepatitis, and A. G., who had compensated Laennec's cirrhosis.

Viewed in another context, the observations recorded in Table I provide additional information. When, as in Figure 1, arterial LGG-hydrolyzing is plotted against hepatic peptidase A–V differences and uptakes, the relationships are seen to be roughly linear and the calculated positive correlation coefficients are significant statistically. It seems evident, then, that the higher the arterial peptidase activity, the greater the removal of enzyme in the splanchnic area and presumably the liver, a finding which is reminiscent of other known homeostatic functions of the latter organ.

DISCUSSION

It should be emphasized that the rate at which a specific peptide substrate is hydrolyzed by crude peptidase preparations, such as plasma, cannot be considered a simple function of the enzyme concentration alone. Enzymic hydrolysis is known to be strongly affected by other influences, such as temperature, pH, substrate concentration, and the presence or absence of activators or inhibitors. The maintenance of constant temperature and pH and the presence of excess substrate minimize these factors as variables, but with the procedures used in this laboratory the relative influences of enzyme concentration and activators and inhibitors remain undetermined. It is for this reason that emphasis is laid on the less specific term "peptidase *activity*," and changes in activity must be interpreted with caution. In the present instance, for example, it would be hazardous to accept the data as evidence that enzyme protein is actually removed from the plasma during its passage through the splanchnic area. The splanchnic A–V differences in peptidase activity which were observed could be due to any one or combination of the following: (1) Actual removal of enzyme protein; (2) intravascular denaturation of enzyme protein; (3) addition of an enzyme inhibitor; (4) removal of an enzyme activator.

The present observations may be interpreted to indicate that the kidney is not a major source of extraction of peptidase activity from plasma. It seems clear, however, that the splanchnic area and presumably the liver does play a role, at least in those individuals with increased plasma LGG-splitting ability, in the removal of this enzymic activity. The latter observations complement those of Fleisher and Butt (11), who found that plasma tripeptidase activity was usually elevated in patients with hepatic disease, especially in the presence of impaired biliary flow. In addition, a high concentration of tripeptidase activity was present in bile obtained after the release of biliary obstruction.

Although the liver is evidently involved in the removal, and perhaps even in the regulation of plasma peptidase activity, a definitive evaluation of this hepatic function will probably require the measurement of hepatic uptake in a normal animal subjected to an intravenous infusion of a concentrated non-toxic peptidase preparation. In these circumstances, the rate of entrance to the plasma would be a known constant and the rate of disappearance the only variable.

SUMMARY AND CONCLUSIONS

1. From acute observations in bilaterally nephrectomized rats, it is concluded that the kidney does not remove appreciable quantities of peptidase activity from the plasma.

2. Peptidase activity is removed from the plasma during its passage through the splanchnic area of patients in whom plasma enzyme levels were found to be elevated. The significant positive correlation obtained between the plasma level and the splanchnic uptake of enzymic activity suggests that the liver is intimately involved in the homeostatic control of these plasma constituents.

ACKNOWLEDGMENT

We wish to thank Drs. W. J. Taylor, R. Kibler, and L. Hamrick for supplying hepatic vein blood samples and Mrs. Rose Boyarsky for valuable technical assistance.

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THE RELATION OF DIETARY PROTEIN CONSUMPTION TO N-15 EXCRETION IN NORMAL SUBJECTS AND IN CUSHING'S SYNDROME UTILIZING N-15 GLYCINE ORALLY AND INTRAVENOUSLY

By K. R. CRISPELL, WILLIAM PARSON, and GARDNER HARDEN[1] with the technical assistance of SARAH BRENT

(From the Department of Internal Medicine, University of Virginia School of Medicine, Charlottesville, Va.)

(Submitted for publication October 5, 1953; accepted November 18, 1953)

In a previous paper (1) we have reported studies on the percentage of N-15 excreted over a twenty-four hour period following the oral administration of N-15 glycine to normal subjects and to patients with Cushing's syndrome in nitrogen equilibrium on diets containing approximately one gram of protein per kilogram of body weight. During this study it was noted that the level of protein intake influenced the percentage of N-15 excreted in the urine, an observation first reported by Sprinson (2) in animals and in a healthy volunteer.

The present paper presents data on the influence of various levels of protein intake on the percentage of N-15 excreted in the urine over a twenty-four hour period following either oral or intravenous administration of N-15 glycine to healthy volunteers. Similar data from a patient with Cushing's syndrome given N-15 glycine orally are also presented.

MATERIALS AND METHODS

The healthy volunteers (J. M., A. W., G. H., P. S., L. G., S. H., J. B., O. B.) were male medical students or house officers between the ages of 21 to 29 years. Patient M. C. was a 24-year old female with Cushing's syndrome shown by adrenal exploration to have adrenal hyperplasia.

The healthy volunteers were fed from the special diet kitchen of the metabolic unit. They carried out their usual laboratory duties while on the study. Patient M. C. was studied while on the metabolic unit.

The diets were arbitrarily defined as follows:

A normal protein diet contained approximately one gram of protein per kilogam of body weight.

A high protein diet contained at least 1.6 grams of protein per kilogram of body weight.

A low protein diet contained a maximum of 0.4 grams of protein per kilogram of body weight.

The amount of nitrogen in the diets is recorded in Tables I and II. When a subject was studied on more than one protein level, the diet was kept isocaloric except as recorded in Table I. An analysis of an aliquot of the diet was made at least once for each study. The nitrogen balance as recorded in Table III was estimated by an analysis of an aliquot of a 24-hour urine specimen by the macro-Kjehldahl technique. The fecal nitrogen was assumed to be 1.3 grams daily (3).

The method of the oral administration of N-15 glycine was as previously described (1). The N-15 glycine for intravenous administration was dissolved in 10 ml. of distilled water and then autoclaved. It was given intravenously over a five-minute period.

The subjects were given the N-15 glycine with the breakfast meal or at breakfast time while in the fasting state (Tables I and II). The fasting state was an overnight fast and the breakfast meal was omitted with the other meals being given at the usual time followed at 9:00 p.m. with the breakfast meal so that the daily nitrogen and caloric intake remained constant. The amount of N-15 administered is recorded in Tables I and II. The subjects were given the N-15 glycine after they had been on a diet for at least one week, and nitrogen equilibrium had been achieved except in a few instances as recorded in Table III.

The amount of N-15 excreted in the urine over a 24-hour period was calculated by determining the N-15 to N-14 ratio of the urinary nitrogen by mass spectrometer analysis. The technique of preparing the samples for the mass spectrometer and the type of instrument was as previously described (1). Results are recorded in Tables I and II as the percentage of administered N-15 excreted in the urine in the first 24-hour period following the administration of the N-15 glycine.

RESULTS

Normal diet

The data on healthy subjects (A. W. and J. M.) were included in a previous paper (1) which reported that healthy young subjects in nitrogen equilibrium, both male and female, on a normal protein intake given N-15 glycine orally with the breakfast meal excreted similar amounts of N-15 over a 24-hour period (25.9 per cent S.D. ± 1.6).

[1] Now at New York Hospital, New York, N. Y.

TABLE I

Oral administration of N^{15} glycine

Subject	Weight Kg.		Nitrogen intake N^{14} 24 hrs. Gm.	Nitrogen intake N^{15} Single dose mg.	Urinary nitrogen N^{14} 24 hrs. Gm.	Urinary nitrogen N^{15} 24 hrs. mg.	Urinary nitrogen N^{15} 24 hrs. %
Healthy volunteers							
Normal diet (with breakfast)							
A. W. No. 1	97.0		16.1	104	14.8	28.0	27.0
No. 2	93.5		16.1	104	13.2	29.3	28.1
J. M.	80.0		12.1	33	11.4	7.6	23.0
Normal diet (Fasting)							
S. H.	76.8		12.1	20.8	10.8	5.4	25.9
High protein diet (with breakfast)							
A. W.	91.6		26.6	78	24.3	35.6	45.3
Low protein diet (with breakfast)							
J. M.	80.0		3.5	33	4.5	2.6	7.8
Cushing's syndrome							
Normal diet (with breakfast)		Calories					
M. C. No. 1	62.9	1970	10.9	78	9.5	37.6	48.2
No. 2	63.7	1970	10.9	39	10.1	20.2	51.7
No. 3	61.5	1970	10.9	22	10.1	10.4	47.3
No. 4	61.0	860	11.0	39	10.2	17.8	45.2
High protein diet (with breakfast)							
M. C. No. 1	61.6	1970	18.0	33	14.7	14.7	44.5
No. 2	59.8	865	18.0	39	14.9	19.9	50.6
Low protein diet (with breakfast)							
M. C. No. 1	62.2	2470	3.5	39	5.1	15.2	39.0
No. 2	63.5	860	3.5	39	4.5	10.7	27.4

Subject S. H. given N-15 glycine orally while in the fasting state also excreted 26 per cent of the N-15 in the urine in 24 hours.

Subjects (G. H., P. S., and L. G.) given N-15 glycine intravenously while fasting excreted an average of 17.2 per cent of the N-15 over a 24-hour period. Subject S. H. excreted 14.1 per cent of N-15 over a 24-hour period when given the N-15 glycine intravenously while consuming the breakfast meal (Table II).

The data on patient M. C. were also included in a previous paper (1) where it was shown that three patients with Cushing's syndrome in nitrogen equilibrium given N-15 glycine orally excreted an average of 47.9 per cent of the N-15 over a 24-hour period (Table I).

High protein diet

Subject A. W. given N-15 glycine orally excreted 45.3 per cent of the N-15 in 24 hours (Table I).

Subjects (O. B., P. S., and J. B.) given N-15 glycine intravenously while fasting excreted an an average of 32.6 per cent of the N-15 in 24 hours (Table II).

Patient M. C. given N-15 glycine orally on the high protein diet isocaloric with the normal protein diet excreted 45 per cent. When tested on a low caloric high protein diet she excreted 51 per cent (Table I).

Low protein diet

Subject J. M. given N-15 glycine orally excreted only 7.8 per cent of the N-15 in 24 hours (Table I).

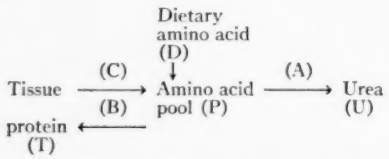
Subjects (O. B. and G. H.) given N-15 glycine intravenously while fasting excreted an average of 5 per cent of the N-15 in 24 hours (Table II).

Patient M. C. on a low caloric diet excreted 27.4 per cent in 24 hours, and on a high caloric diet excreted 39.0 per cent in 24 hours with the N-15 glycine being administered orally on both occasions (Table I).

DISCUSSION

It is clear that nitrogen equilibrium is quite readily achieved when protein consumption is varied widely. Classical nitrogen balance studies do not offer any explanation of the change in the "metabolic set" with respect to nitrogen metabolism. These studies utilizing N-15 offer some partial insight into the alteration in the handling of nitrogen.

The pertinent results are summarized in Tables I and II and show clearly that the N-15 excretion in the urine after either oral or intravenous administration of N-15 glycine is profoundly influenced by the previous level of protein intake in the diet. A high protein diet causes an increase and a low protein diet a decrease in the amount of N-15 excreted over a twenty-four hour period as compared to a normal protein diet.



As shown in the above simplified version of the diagram used by Rittenberg (4), dietary amino acids (D) may be thought of as entering an amino acid pool (P). A portion is catabolized (A) to urea (U) and a portion is used to build tissue protein (T). According to the present concept of the dynamic state there is a continuous synthesis (B) of tissue protein (T) associated with a continuous breakdown (C). Under conditions of nitrogen equilibrium, B = C. It is obvious that both the amino acids from the diet and from the breakdown of tissue protein contribute to the amino acid pool.

The most reasonable explanation for the large increase in the amount of N-15 appearing in the urine on a high protein diet is an increase in the rate of reaction A. More specifically there is an increased rate of degradation of dietary glycine to urea. The increased excretion of N-15 is not due to an increased excretion of glycine *per se* because in both the normal and high protein diets approximately ninety per cent of the N-15 appears in the urea fraction. Also, Eckhardt and Davidson (5) have shown that a large fluctuation in the type and quantity of protein eaten results in only small changes in the quantity of amino acids excreted. The possibility that there is a significant increase in the rate of reaction C can be ruled out since the N-15 incorporated into tissue protein would be greatly diluted with normal nitrogen (N-14) and even a great increase in the degradative rate (C) would result in a very small amount of the N-15 being excreted in twenty-four hours.

The decrease in the amount of N-15 appearing in the urine on a low protein diet could be due either to a decrease in the rate of reaction A or to

TABLE II

Intravenous administration of N^{15} glycine

Subject	Weight *Kg.*	Nitrogen intake		Urinary nitrogen		
		N^{14} 24 hrs. *Gm.*	N^{15} Single dose *mg.*	N^{14} 24 hrs. *Gm.*	N^{15} 24 hrs. *mg.*	N^{15} 24 hrs. %
Healthy volunteers						
Normal diet						
(Fasting)						
G. H.	77.7	12.3	30	10.9	5.35	17.8
P. S.	71.8	11.8	30	9.5	4.85	16.2
L. G.	77.8	12.7	60	11.3	10.56	17.6
Normal diet						
(with breakfast)						
S. H.	75.3	12.0	30	10.6	4.24	14.1
High protein diet						
(Fasting)						
J. B.	70.0	27.9	24	27.7	8.60	35.8
O. B.	71.6	28.6	30	26.0	9.75	32.5
P. S.	71.6	23.0	30	22.2	8.88	29.6
Low protein diet						
(Fasting)						
O. B.	72.0	4.1	30	3.5	1.54	5.1
G. H.	77.6	4.2	30	2.9	1.45	4.8

TABLE III

Oral administration of N^{15} glycine

Subject	Wt. range Kg.	Daily nitrogen balance: Days before tag 5	4	3	2	1	Days after tag 1	2	3	4	5
Healthy volunteers											
Normal protein diet											
A. W. No. 1	95.4–97.7	−1.2	−1.4	−0.2	−0.3	−1.0	−0.2	+0.2	+0.4	+0.7	−1.1
No. 2	91.8–93.6	−0.2	−0.1	−0.2	−0.1	−0.1	−0.1	−0.7	−0.5	—	—
J. M.	80.0–80.1	−3.8	−3.3	−2.1	−1.2	−0.3	−0.6	−0.3	+0.4	+0.1	—
S. H.	76.8–76.9	−1.2	−0.4	−0.2	+0.1	0.0	0.0	—	—	—	—
High protein diet											
A. W.	91.9–90.0	—	—	+3.7	+2.2	+1.2	+0.8	+0.4	−0.2	—	—
Low protein diet											
J. M.	80.0–80.4	−2.5	−2.8	−3.7	−1.6	−1.2	−2.3	−2.3	−2.4	—	—
Cushing's syndrome											
Normal protein diet											
M. C. No. 1	62.8–63.6	—	+0.5	−0.1	−0.4	+1.4	+0.1	+0.4	+0.3	+0.3	+0.6
1970 cal. No. 2	63.5–63.8	−0.1	−0.2	−0.4	−0.4	−0.3	−0.5	−0.7	+0.6	+0.2	−0.7
No. 3	61.3–61.6	0.0	+0.2	−0.4	−0.5	−0.4	−0.5	−0.4	0.0	−0.2	—
860 cal. No. 4	61.3–61.0	+0.7	+0.7	−0.6	—	+0.6	−0.5	+0.3	−0.1	+1.3	−0.2
High protein diet											
865 cal. No. 5	60.1–60.4	—	+3.3	+1.0	+1.2	+1.7	+1.8	+0.4	−0.5	−0.1	+0.5
1970 cal. No. 6	60.0–59.7	+1.6	+1.3	+1.1	+2.4	+0.6	+1.8	+1.5	+2.4	+0.9	—
Low protein diet											
860 cal. No. 7	62.2–61.6	−1.7	−2.4	−2.8	−2.4	−2.0	−2.3	−2.0	−1.6	−1.2	−1.3
2470 cal. No. 8	63.6–63.8	−1.1	−1.1	−1.1	−1.3	−1.0	−1.1	−1.1	−1.6	−1.2	−1.3
Intravenous administration of N^{15} glycine											
Normal protein diet											
P. S.	70.7–71.6	−0.9	−0.2	−0.9	+0.3	+0.4	+1.0	—	—	—	—
G. H.	77.7–78.1	+1.6	+2.2	+0.9	+0.1	+0.4	+0.1	—	—	—	—
L. G.	77.7–77.9	−1.2	−0.4	−0.5	−0.2	+0.1	−0.6	−0.4	—	—	—
S. H.	74.5–76.0	−0.1	+0.1	−0.1	−0.3	−0.3	+0.2	+0.6	+1.0	+1.4	+0.9
High protein diet											
O. B.	71.6–71.8	+1.1	+2.5	+0.9	+2.2	+1.3	+0.3	+2.1	+2.2	—	—
P. S.	71.6–71.9	+2.8	+1.3	+0.6	+1.1	+1.4	−0.6	—	+1.5	—	—
J. B.	70.0–70.1	+0.7	−1.1	−0.2	0.0	−0.5	−1.1	—	−0.8	+0.4	—
Low protein diet											
G. H.	77.6–78.2	−1.7	−1.4	−1.3	−0.7	−1.3	0.0	+0.7	−1.4	−0.1	−0.6
O. B.	71.9–72.0	−2.5	−1.9	−0.7	−1.4	−0.4	−0.7	−1.8	−0.4	−0.7	—

an increase in the rate of reaction B. The data do not permit differentiation between these two possibilities.

Of interest also is the fact that there was a decreased percentage of N-15 excreted following intravenous as compared with oral administration.

In animal experiments with amino acid mixtures several investigators have demonstrated that tissue anabolism occurs only when all the essential amino acids are supplied simultaneously (6, 7). A time interval any greater than one hour between the ingestion of an incomplete amino acid mixture and its supplementation with the missing amino acids resulted in the failure of tissue synthesis (6). In this study with a non-essential amino acid no difference in N-15 excretion was observed when a comparison was made of the experiments when the N-15 glycine was given concurrently with breakfast with those where the food was not ingested until four hours after the N-15 glycine was given.

Utilization of the technique of San Pietro and Rittenberg (8), which permits a mathematical calculation of the data and derivation of synthesis rates, is underway using both tagged essential and non-essential amino acids and may offer further information regarding utilization of amino acids.

We have previously reported (1) that patients with Cushing's syndrome in nitrogen equilibrium excrete approximately 48 per cent of the N-15 in 24 hours as compared to about 26 per cent in normal subjects. The data suggested that this represented decreased protein synthesis and increased

degradation and excretion of ingested amino acids. Clark (9) has recently reported observations in animals which are in accord with this concept of the action of cortisone on protein metabolism.

Patient M. C., with Cushing's syndrome on a high protein diet did not excrete a significantly increased amount of N-15 (50.5 per cent) as compared to the normal diet (47.9 per cent). This suggests that in Cushing's syndrome the degradation of ingested amino acids was at a "maximum" and was not further increased by increasing the dietary protein.

SUMMARY AND CONCLUSIONS

1. The percentage of N-15 excreted in the urine over a 24-hour period following either the oral administration or intravenous injection of N-15 glycine has been determined in healthy subjects on various levels of protein intake in approximate nitrogen equilibrium.

2. An increased level of protein intake caused an increased excretion of N-15 in the urine and a decreased protein intake caused a decreased excretion of N-15 in the urine. The pattern of excretion was the same whether the N-15 glycine was given orally or intravenously, although the percentage excreted was lower after the intravenous route.

3. The excretion of N-15 was not influenced by the food being ingested concurrently with glycine N-15 administration or ingested four hours later.

4. When a patient with Cushing's syndrome was put on a high protein diet the urinary excretion of N-15 was not increased from what it had been on a normal protein intake.

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THE VARIATIONS OF THE SODIUM SPACE AND THE TOTAL EXCHANGEABLE SODIUM DURING PREGNANCY [1]

By MARY JANE GRAY AND ALBERT A. PLENTL

(From the Department of Obstetrics and Gynecology, Columbia University, College of Physicians and Surgeons, and The Presbyterian Hospital, New York, N. Y.)

(Submitted for publication September 14, 1953; accepted November 25, 1953)

Excessive sodium retention has been implicated as a primary or contributing factor in the etiology of pre-eclampsia and evidence to this effect is said to be well substantiated. An increase in serum sodium concentrations during such abnormal pregnancies has never been demonstrated and it is, therefore, concluded that sodium is stored by expansion of the extra-cellular space or incorporated in selected cell systems.

Careful sodium balance studies (1–5) on limited numbers of normal pregnant patients at isolated gestational periods have also pointed to a persistent retention of sodium, which would lead to the suspicion that there exists a quantitative rather than qualitative difference between normal and pre-eclamptic patients. A definition of the expected variations in the sodium content of normal pregnant patients would be an essential base line for further investigations on the sodium metabolism in abnormal pregnancies.

The present report relates to a study of the total exchangeable sodium and the sodium space by the isotope dilution method. It was undertaken in an effort to define the limits of normal variations for uncomplicated pregnancies.

METHODS [2]

Because of the potential hazard involved in the use of radioactive materials in pregnant women, the number of determinations performed during this study was sharply limited. The dosage used was 30 microcuries of Na^{24} per determination per patient, at minimum intervals of four weeks not exceeding six such determinations per antepartum course. On the third or fourth postpartum day and again four weeks after delivery, the determinations of sodium space and total exchangeable sodium were repeated.

After a control sample of blood was drawn from the patient, an infusion of 5 per cent dextrose in water was started. An accurately known volume of the tracer solution containing approximately 30 microcuries of Na^{24} was allowed to flow into the infusion tubing fom a 25 ml. analytic burette by a method previously described (6). Blood samples were then drawn at 3, 5, and 24-hour intervals. The urine was collected over the same 24-hour period.

The unused portion of the solution remaining in the burette was collected and set aside for comparison and determination of the total dose administered. In order to bring the test solution within the appropriate range of the analyzing instrument, a dilution of the specimens to 1:200 was found to be most satisfactory. One-half ml. portions of this diluted tracer solution, 0.5 ml. of the 3, 5, and 24-hour serum samples and 2 ml. aliquots of pooled urine were pipetted into planchets in triplicate and dried under an infra-red heat lamp. Because of the low dosage administered, the counting was performed by means of a Nuclear Q-gas counter. The number of counts averaged over 300 per minute and was in excess of ten times the background. All counts were corrected for background and radioactive decay as well as serum absorption. The sodium space was calculated from average values obtained on 3 and 5-hour sera and a knowledge of the total number of counts administered by the familiar formula:

$$\text{Sodium Space in ml.} = \frac{(\text{count/sec./ml. of test sol.})(\text{ml. injected})}{\text{counts/sec./ml. of serum}}$$

For the calculation of the 24-hour sodium space, the total radioactivity of the 24-hour urine was subtracted from the number of counts injected. The 24-hour sodium space multiplied by the concentration of sodium in the serum (mEq. per 1000 ml.) gave the "total exchangeable sodium" in milliequivalents. The sodium content of the 24-hour serum was estimated by the flame-photometric technique.

CLINICAL MATERIAL

Ten primigravidae were chosen for this study on the basis of a normal history, physical examination, and routine laboratory tests. An effort was made to use volunteers who registered during the first trimester, in order to obtain experimental data over as long a period as pos-

[1] This investigation was supported by a Research Grant from the National Institutes of Health, Department of Health, Education and Welfare.

[2] The authors are indebted to Dr. E. Quimby of the Department of Radiologic Research for her unfailing cooperation, advice and assistance during all phases of the investigations here presented.

sible and to provide a base line for future, anticipated changes. One of these patients developed signs and symptoms of mild pre-eclampsia during her third trimester, and underwent Cesarean section at term because of cephalo-pelvic disproportion. Two patients had short episodes of hyperemesis gravidarum during the first trimester, and one was hospitalized near term because of nausea and vomiting. The six remaining patients had entirely uncomplicated antepartum courses. All infants were normal, and none of the mothers had any complication during the puerperium.

RESULTS

Most of the data obtained on these patients are reproduced in Table I. All patients had normal weight gains according to the range set forth by Chesley (7). Although there is an absolute gain in sodium space from the beginning of pregnancy until term, the values show little variation when expressed as per cent of body weight. Only the pre-eclamptic patient, B. D., showed a significant increase in sodium space expressed as per cent of body weight, during the third trimester.

The absolute values for the total exchangeable sodium show a similar, though not exactly parallel, increase during pregnancy, but when these figures are expressed as milliequivalents per kilogram they remain essentially unchanged.

TABLE I

The absolute and relative values of sodium space and total exchangeable sodium and their changes during normal and pathologic pregnancies

Patient	Week of gestation	Weight	Sodium space	Sodium space	Total exchangeable sodium	Total exchangeable sodium	Total exchangeable sodium	Sodium space
		Kg.	*L.*	*% body wt.*	*mEq.*	*mEq./Kg.*	*mEq./m.²*	*L./m.²*
B. W.	10	80.0	18.5	23.1	2606	32.6	1316	9.34
	16	77.4	18.5	23.9	3553	45.9	1813	9.42
	22	86.4	21.8	25.2	3470	40.2	1700	10.66
	28	85.5	19.9	23.3	3311	38.8	1631	9.80
	33	87.7	20.8	23.7	3449	39.3	1674	10.10
	38	91.7	20.6	22.5	3146	33.3	1491	9.76
	2 days p.p.	87.1	21.1	24.2	3052	35.1	1489	10.29
	1 mo. p.p.	86.1	21.0	24.4	3555	41.3	1743	10.29
J. B.	10	65.5	16.1	24.7	2489	38.0	1439	9.34
	16	64.3	16.2	25.2	2507	39.0	1458	9.42
	22	66.4	16.3	24.6	2340	35.3	1336	9.31
	27	68.2	17.0	24.9	2760	40.5	1564	9.60
	32	70.9	16.2	22.9	2934	41.4	1621	8.90
	38	72.1	18.0	25.0	3063	42.5	1683	9.89
	3 days p.p.	65.1	14.1	21.7	2232	34.6	1290	8.15
	1 mo. p.p.	62.7	15.9	25.5	2599	41.4	1529	9.35
R. C.	10	68.6	15.9	23.2	2355	34.3	1346	9.09
	16	73.6	17.3	23.5	2510	34.0	1233	9.44
	22	78.2	18.8	24.0	3164	40.5	1674	9.92
	30	82.3	19.4	24.8	2959	36.0	1533	10.03
	38	83.4	19.8	25.3	2859	34.3	1474	10.23
	3 days p.p.	76.8	18.4	23.9	2874	37.4	1545	9.87
	1 mo. p.p.	73.6	16.0	21.7	2283	31.0	1248	8.74
S. S.	12	52.3	13.6	26.0	2286	43.7	1504	8.95
	18	56.1	13.0	23.2	2008	35.8	1287	8.33
	24	58.4	14.4	24.7	2526	43.3	1588	9.08
	30	60.2	13.1	21.8	2227	37.0	1392	8.22
	35	61.1	13.8	22.5	2213	36.2	1374	8.54
	*39	59.8	15.8	26.4	2085	34.9	1303	9.88
	4 days p.p.	55.2	12.9	23.4	1971	35.7	1272	8.32
	1 mo. p.p.	54.0	12.5	23.2	1928	35.7	1252	8.12
G. S.	16	53.2	14.9	28.1	2142	40.3	1400	9.74
	21	55.5	16.4	29.5	2442	44.1	1536	10.55
	26	57.8	15.2	26.2	2586	44.7	1626	9.53
	30	57.1	15.1	26.4	2663	46.7	1696	9.59
	3 days p.p.	56.1	12.6	22.4	2355	42.0	1510	8.08
	1 mo. p.p.	54.3	14.4	26.5	2647	48.7	1713	9.35

* Patient hospitalized because of nausea and vomiting.

TABLE I—*Continued*

Patient	Week of gestation	Weight	Sodium space	Sodium space	Total exchangeable sodium	Total exchangeable sodium	Total exchangeable sodium	Sodium space
		Kg.	*L.*	*% body wt.*	*mEq.*	*mEq./Kg.*	*mEq./m.²*	*L./m.²*
J. W.	13.0	57.2	12.4	21.7	2077	36.3	1340	8.00
	19	56.7	14.5	25.6	2257	39.8	1456	9.35
	25	55.5	14.5	26.2	2278	41.1	1488	9.48
	31	60.0	15.9	26.4	2969	49.5	1856	9.91
	37	61.7	16.6	26.9	2588	41.9	1598	10.25
	3 days p.p.	54.8	16.6	27.6	2502	45.7	1625	10.78
	1 mo. p.p.	55.0	14.4	26.1	2208	40.1	1434	9.32
T. M.	11	64.1	13.7	21.4	2317	38.0	1371	8.10
	*15	59.7	13.7	22.9	1905	31.9	1161	8.35
	21	61.8	17.0	27.5	2442	39.9	1490	10.30
	27	64.3	17.1	26.5	2960	46.9	1751	10.09
	32	65.6	18.3	27.7	2905	44.3	1689	10.55
	37	68.2	18.2	26.7	2923	42.9	1680	10.46
	3 days p.p.	65.3	15.3	23.3	2321	35.5	1349	8.89
	1 mo. p.p.	59.6	12.6	21.6	1990	33.4	1170	7.68
J. P.	11	55.9	12.7	22.7	2057	36.8	1327	8.19
	17	57.1	13.2	23.2	2232	39.1	1431	8.47
	23	59.3	14.6	24.5	2615	44.1	1655	9.22
	27	60.5	14.1	23.3	2485	41.1	1563	8.87
	32	61.5	14.4	23.3	2286	37.2	1429	8.97
	37	61.1	15.6	25.5	2407	39.4	1504	9.75
	4 days p.p.	56.6	13.4	23.6	1948	34.4	1249	8.56
	1 mo. p.p.	56.9	12.2	21.4	2323	40.8	1489	7.82
J. L.	13	59.1	14.4	24.4	2271	38.4	1446	9.17
	18	62.5	15.6	24.9	2431	38.9	1473	9.66
	25	66.3	14.0	21.1	2240	33.3	1358	8.48
	30	69.6	14.8	21.3	2530	36.4	1479	8.76
	34	73.5	14.2	19.3	2604	35.4	1505	8.21
	38	75.2	17.7	23.5	2876	38.2	1643	10.11
	4 days p.p.	70.2	14.0	19.9	2182	31.1	1291	8.28
	1 mo. p.p.	66.4	13.2	19.9	1956	31.6	1185	8.00
B. D.	23	67.3	18.3	27.2	2443	36.3	1420	10.64
	27	68.0	20.2	29.6	3196	47.0	1847	11.68
	31	70.3	22.4	31.8	3147	44.7	1798	12.77
	35	70.9	—	—	3942	55.6	2240	—
	†39	70.9	23.8	33.6	3699	52.2	2102	13.52
	3 days p.p.	61.4	15.0	24.5	3131	51.0	1694	9.09
	1 mo. p.p.	60.2	—	—	2762	45.9	1694	—

† Patient hospitalized because of signs and symptoms of pre-eclampsia.

The values for sodium space and total exchangeable sodium are also expressed per square meter of body surface, a terminology used by other authors (8); this manner of presentation is said to be particularly advantageous in investigations on the physiology of pregnancy.

Table II summarizes the data by trimester, their sum, and the puerperium but excludes the data obtained on the pre-eclamptic patient. The sodium space throughout pregnancy averaged 24.4 per cent of body weight, with a range of 19.3 to 29.5 per cent. The total exchangeable sodium averaged 39.3 mEq. per Kg. with a range from 31.9 to 49.5 mEq. per Kg. The corresponding values for the pre-eclamptic patient were within normal limits during the second trimester but showed a significant rise during the early part of the third trimester, preceding the time at which clinical evidence for pre-eclampsia developed. The gain in total exchangeable sodium during pregnancy and its loss during the puerperium for the six entirely normal patients are presented in Table III. These patients gained an average of 510 mEq. of sodium during an average of 26 weeks of pregnancy and lost an average of 440 mEq. of sodium during the puerperium.

The hyperemetic patients (T. M., B. W., and S. S.) and the patient who eventually developed

TABLE II

The average values for the total exchangeable sodium and the sodium space of six normal patients arranged according to trimesters and the postpartum period

Period	Total exchangeable sodium	Sodium space as % of body weight
	mEq./Kg.	
1st trimester	37.3 (32.6–43.7)	23.4 (21.4–26.0)
2nd trimester	39.9 (31.9–46.0)	24.9 (21.1–29.5)
3rd trimester	39.2 (33.3–49.5)	24.2 (19.3–27.7)
All trimesters	39.3 (31.9–49.5)	24.4 (19.3–29.5)
Postpartum	37.5 (31.0–48.7)	23.3 (19.9–27.6)

pre-eclampsia (B. D.) were not included in Table III. Preceding and during episodes of hyperemesis these patients lost appreciable amounts of sodium as shown by a drop in the total exchangeable sodium which, after treatment, returned to normal limits.

COMMENT

The demonstration by Moore (9) that it is possible to calculate the total amount of an element in the body provides a means of studying metabolic processes. The application of this principle to electrolyte metabolism can yield indirect information on the intake and output of these elements, and thus serve as a metabolic balance study.

TABLE III

Calculation of the average gain and loss of the total exchangeable sodium during the ante- and postpartum period based upon the data obtained on six entirely normal patients

Patient	Period of gestation	Number of weeks	Milliequivalents of total exchangeable sodium	
			Gained	Lost
	Weeks			
J. W.	13th–37th	24	511	—
	Puerperium	4	—	380
J. P.	11th–37th	26	350	—
	Puerperium	4	—	184
J. L.	13th–38th	25	605	—
	Puerperium	4	—	920
J. B.	10th–38th	28	574	—
	Puerperium	4	—	464
R. C.	10th–38th	28	504	—
	Puerperium	4	—	576
G. S.	16th–30th	24	521	—
	Puerperium	4	—	16
Average		25.8	510	440
Average per week			19.8	

The values obtained for the total exchangeable sodium over the whole period of normal gestation are roughly of the same magnitude per unit body mass as those obtained by other authors for non-pregnant, normal, female subjects. To date a total of only 16 such determinations were carried out on female subjects as reported by Forbes and Perley (10), Miller and Wilson (11), and other investigators (12). The results do not differ materially from normal male subjects if they are expressed in milliequivalents of sodium per kilogram of body weight (10–13). All of these values except one, reported by Forbes and Perley (54.1 mEq. per Kg.), are within a range of 2 σ of any set of values here reported.

Comparing the changes in weight recorded on the 10 patients here investigated, all fall well within the expected range (7), whether the weight one month postpartum is taken as a norm or the patient's stated pre-pregnancy weight is used as a point of reference. Even the pre-eclamptic patient gained weight only within these limits which is to be expected because of the reported lack of correlation of weight gain and incidence of toxemia. In addition to the net gain these weight changes also showed the expected progression except for the three subjects who were hospitalized because of excessive vomiting.

The loss of water, although not directly determined, is presumably reflected in the loss of weight observed on the three patients in question (T. M., B. W., and S. S.). Because of inadequate nutrition, these patients were in a negative sodium balance, hence the total exchangeable sodium would be expected to decline. A substantial fall in the absolute and relative values for the total exchangeable sodium substantiates this deduction. The loss of sodium is proportionately less than the loss of water and chloride and a decline in extracellular anions should then cause a similar decline in ca tions if a state of compensated acidosis is to be maintained. A relatively small drop in the serum sodium concentration, if reflected in the whole extracellular fluid, should cause an apparent, but not real, expansion of the fluid volume of distribution of sodium. This affords an explanation for the

striking increase in sodium space, not only in its relative, but also in its absolute values in the face of a pronounced fall in total exchangeable sodium.

In contrast to the positive rate of gain in weight which reaches its maximum value at term, the rate of retention of sodium is inconsistent. Maximal sodium retention may occur any time during the second trimester, but the net gain, regardless of fluctuations, appears to be reasonably constant. The six entirely normal patients gained an average of 510 mEq. of exchangeable sodium with an average gain of about 20 mEq. per week.

This retention of sodium is considerably less than predicted by other authors. Freyberg, Reekie, and Folsome (5) reported the lowest value for the weekly retention of sodium during the latter part of pregnancy, namely 1.61 Gm. (70 mEq.). Thompson and Pommerenke (1) and Taylor, Warner, and Welsh (2) reported an almost identical value of 3.08 Gm. (134.0 mEq.) gain per week, a value which agrees with the data reported by Hummel, Steinberger, Hunscher, and Macy (3), who thought that the gain amounted to 3.92 Gm. (170 mEq.) per week. Coons, Coons, and Schiefelbusch (4) concluded that the average weekly gain was 8.81 Gm. (383 mEq.).

The lowest of these values (5) was determined over the last 70 days of the period of gestation. This corresponds to a daily retention of 0.231 Gm. of sodium or a total of 16.3 Gm. for these 70 days of investigation. The rate of gain of sodium according to Taylor, Warner, and Welsh (2) near the end of pregnancy amounted to 0.440 Gm. per day or 26.4 Gm. in two months. This would mean that a normal patient would increase her total sodium content by an additional 45 per cent during the last two months of pregnancy, and, if the figures of Coons, Coons, and Schiefelbusch (4) are accepted, she would have to double her sodium content during that time.

While most of these studies were carried out during the latter part of pregnancy, one group of investigators (Thompson and Pommerenke [1]) also reported a value of 0.440 Gm. per day for the fourth month of gestation. If this figure were accepted, it would imply that the sodium content of an average patient would more than double during the last two trimesters.

The data here reported indicate that only 12.0 Gm. of sodium (510 mEq.) were retained during the last two trimesters. About one-half to two-thirds of this amount can be accounted for in the products of gestation, and the remainder is to be sought in the expanded maternal blood volume. The concentration of sodium in the amniotic fluid is nearly equal to that of plasma, and, since the average volume of the amniotic fluid in term pregnancies is about 850 ml. (14), this would contain about 110 mEq. of sodium. The sodium space of term infants according to Perley, Forbes, and Pennoyer (15) is about 35.2 per cent of body weight, which, for a plasma sodium concentration of 135 mEq., would correspond to a total exchangeable sodium of 47.5 mEq. per Kg. A term baby weighing 3200 Gm. would, therefore, contain about 152 mEq. of exchangeable sodium. If during pregnancy the maternal plasma had expanded by 1500 ml., this would, under conditions of unchanged sodium concentration, account for the remainder.

The fact that an almost equivalent amount of sodium is lost during the delivery and the puerperium, further demonstrates the correctness of these calculations and experimental data.

A possible explanation for the discrepancy between these data and those of others may be sought in the fact that in the present study only the exchangeable sodium has been determined while the classical balance studies consider the net gain in the sum of exchangeable and permanent sodium. One would have to assume that enormous amounts of sodium are stored in bone in a non-exchangeable form, and direct evidence to this effect does not exist.

Numerous attempts have been made to determine the extracellular fluid space and its changes during pregnancy. The distribution of thiocyanate, bromide, radio-sodium, and inulin have been used for this purpose. By far the largest number of determinations based on the distribution of thiocyanate have been reported by Chesley and Chesley (16, 17), but these results cannot easily be compared with those obtained by others. A value of 28.2 per cent of body weight reported by Freis and Kenny (18) is in good agreement with the findings of Caton, Roby, Reid, and Gibson (8). The application of the bromide dilution method gave similar results in the hands of Friedman, Goodfriend, Berlin, and Goldstein (19). So far only two reports on the use of radio-sodium have appeared,

and both indicate that there is no appreciable difference between the sodium space and the thiocyanate space. Nineteen such analyses by Cox and Chalmers (20), gave an average of 27.9 per cent of body weight during the last trimester, and Lambiotte-Escoffier, Moore, and Taylor (21), found a sodium space of 26 and 29.6 per cent of body weight on two normal pregnant subjects.

Only the data of Caton, Roby, Reid, and Gibson (8) include the earlier phases of pregnancy. In order to compare results here presented with those obtained by others, it would be necessary to limit this comparison to the third trimester, where the values here reported give an average of 24.3 with a standard deviation of 2.2. One can, therefore, assume that the sodium space of 95.5 per cent of all normal patients falls within a range of 19.9 to 28.6 per cent of body weight. This represents a much narrower range than that reported by Cox and Chalmers (20), which may well be due to the uniformity of parity and age of our subjects.

In the calculation of average values, the data obtained on patient D. B. were excluded because of clinical signs and symptoms of pre-eclampsia. The obvious increase of the total exchangeable sodium in this patient beyond the established limits for normal pregnant patients suggests that sodium is retained, but does not permit a differentiation between an expansion of the volume of distribution due to an increase in intracellular sodium or an expansion of the extracellular fluid space (21).

SUMMARY

Ten primigravidae of comparable age were followed throughout their pregnancies by frequent determination of sodium space and total body sodium. Two of these patients had short periods of hyperemesis gravidarum, one was hospitalized near term because of vomiting and one developed signs and symptoms of mild pre-eclampsia.

The relative values for sodium space and total exchangeable sodium did not change appreciably for each of the normal patients during the period of observation. An average value of 24.4 per cent (range 19.3 to 29.5) of body weight was obtained for the sodium space, and the total exchangeable sodium was found to be 39.3 (range 31.9 to 49.5) milliequivalents per kilogram of body weight.

The total exchangeable sodium of the patient who eventually developed pre-eclampsia remained within normal limits during the second trimester, but exceeded the statistical limits in the early part of the third trimester, at which time no clinical evidence for pre-eclampsia existed.

The six normal patients showed an average absolute gain in total exchangeable sodium of 20 milliequivalents per week, or a total of 500 milliequivalents during the second and third trimester. This increment in exchangeable sodium could be accounted for in the products of gestation and the expanded maternal blood volume. No evidence for retention of exchangeable sodium was obtained.

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QUANTITATIVE STUDIES ON EXCRETION OF POLIOMYELITIS VIRUS: A COMPARISON OF VIRUS CONCENTRATION IN THE STOOLS OF PARALYTIC AND NON-PARALYTIC PATIENTS [1]

By ROBERT WARD, GERALD A. LoGRIPPO, IRVING GRAEF, AND DAVID P. EARLE, JR.

(From the Departments of Pediatrics and Medicine, New York University College of Medicine, New York, N. Y.)

(Submitted for publication August 6, 1953; accepted November 25, 1953)

During the course of another study (1) it was necessary to determine the concentration of poliomyelitis virus excreted in the feces of patients. Stool specimens were obtained from two groups of patients designated for the present as Groups I and II. The purpose of this paper is to report observations on the titer of virus found in the stools of patients with poliomyelitis. The titer was generally higher in paralytic patients as compared to nonparalytics and to patients with mild transitory weakness. Certain differences in the composition of the two groups of patients and in the handling of the stools from each group became apparent after the titrations were done and will be discussed.

MATERIAL AND METHODS

Material for this study was obtained from patients with poliomyelitis admitted to Willard Parker [2] and to Bellevue Hospitals [2] in their first week of illness during the 1949 epidemic in New York City. The age of the patients varied from 6 months to 22 years (Table I).

The first stool specimen (A) was obtained during the first week of illness, and a second specimen (B) was collected 7 days later. They were stored in the solid state (− 30° C.) until prepared for inoculation.

Preparation of stool for inoculation. The stool was thawed and mixed in a Waring Blendor with sterile distilled water to make a 10 per cent suspension. The suspension was centrifuged at 4000 r.p.m. for 30 minutes at 4° C. (International Centrifuge PRl, angle head). The supernatant fluid was removed and shaken briefly with one-fourth to one-half volume of reagent ether, and stored overnight at 2° C. The next morning, after removal of the ether, the suspension was centrifuged again as described above. The middle aqueous layer was pipetted off and centrifuged at 18,000 r.p.m. for 1 hour at 4° C. (International, PRl, angle head). To the final supernatant fluid penicillin 1000 units per ml. and streptomycin 10 mg. per ml. were added. Portions of the material were cultured on a blood agar plate and inoculated into monkeys. Aliquots were stored at − 30° C.

Inoculation of monkeys. Immature rhesus monkeys (*macaca mulatta*) weighing usually 5 to 7 pounds were each given 1 ml. of stool suspension by the intracerebral route under ether anesthesia. They were examined daily for 28 days. At that time, if no signs of poliomyelitis had appeared, they were sacrificed and the spinal cord and medulla examined histologically. Monkeys exhibiting microscopic lesions typical of poliomyelitis were counted as positive. The diagnosis in animals which had developed weakness or paralysis during the observation period was confirmed also by microscopic examination of the spinal cord.

Titration in monkeys. The 10 per cent suspension of stool was first inoculated into a group of six monkeys as described above. If none developed poliomyelitis, the stool was considered negative. If one or more animals acquired the disease, an aliquot of this original suspension (10^{-1}) was thawed and serial decimal dilutions in sterile distilled water were made through 10^{-6}. Six monkeys were inoculated with each dilution which was prepared just prior to injection and held in an ice bath. If an end point was not achieved by this procedure additional dilutions of 10^{-5} or 10^{-6} were prepared from the original suspension and similarly inoculated.

Titration in tissue culture. Some of the stool suspensions were also titrated in tissue culture. The roller tube culture method described by Robbins, Weller, and Enders (2) was used with certain modifications (3–7). Monkey (rhesus or cynomolgus) testicular tissue provided the source of fibroblastic outgrowth. Eight to ten fragments were placed on the wall of each tube. Nutrient fluids of varied composition were used. The combination of Hanks-Simms medium and lactalbumin hydrolysate as described by Melnick and Riordan (8) proved to be satisfactory. After 6 or 7 days when the growth of fibroblasts was usually adequate, the nutrient fluid was removed from the tubes. Serial decimal dilutions of stool in fresh medium were prepared through the range 10^{-2} to 10^{-7}. Of each dilution, 1 ml. was inoculated into each of three tubes. The tubes were rolled at 6 revolutions per hour at 35° C. They were examined frequently during 8 to 10 days for the cytopathic changes first reported by Robbins, Enders, and Weller (9) as characteristic of poliomyelitis virus. Widespread granulation and destruction of fibroblasts

[1] Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

[2] We thank the Medical and Nursing staffs of Willard Parker Hospital and Bellevue Hospital for their help in collecting material.

TABLE I

Concentration of poliomyelitis virus in stools of two groups of patients determined by titration in monkeys

Group I					Group II				
		Illness					Illness		
Patient	Age	Type*	Day of	Titer†	Patient	Age	Type*	Day of	Titer
	(*Years*)					(*Years*)			
Biv	5	NP	2	4,000	Rom	1.5	P	3	20,000
Sap	7	P	4	500	Apo	8	P	3	16,000
Win	6	P	5	500	Bra	7	P	3	10,000
Mun	2	P	4	400	Cav	0.5	P	6	4,000
Mol	8	Wk	6	40	DeA	1.5	P	5	3,000
Kur	22	Wk	5	30	Cas	10	P	5	2,000
Mag	7	NP	2	16	Con	12	P	3	500
Moo	5	Wk	4	16	Bow	6	NP	6	4
Kun	15	Wk	2	10	Sta	11	Wk	5	4
Nix	7	P	3	5					
Mar	8	NP	4	4					
Lis	10	NP	5	4					

* NP = non-paralytic; P = paralytic; Wk = mild transitory paralysis or weakness.

† Titer is expressed as the reciprocal of the dilution of stool causing infection in 50 per cent of the animals (or culture tubes) inoculated. This figure indicates the number of infectious doses of virus per gram of stool.

(designated as 3 plus or more) was required as evidence of the effect of virus (6a). The 50 per cent end point of titrations performed in roller tubes and in monkeys was calculated by the method of Reed and Muench (10). The end point is expressed in the tables as the reciprocal of the dilution causing infection in 50 per cent of the animals or culture tubes inoculated and indicates the number of infectious doses of virus per gram of stool. The resulting titers in monkeys and in tissue culture tubes are thereby made comparable. Neutralization tests were carried out in tissue culture (6a) on 3 strains which proved to be Type 1.

There were certain differences in storage time and in the technique of processing stools of the two groups; specimens in Group I were kept frozen for a shorter period, *e.g.*, for a few weeks to 18 months, than those in Group II which were frozen for about 30 months. The stools of both groups were prepared and inoculated in the same laboratory by the same method but by different hands.

RESULTS

Table I shows the titer, as measured in monkeys, of poliomyelitis virus in the stools of patients in Group I compared with those in Group II. The majority (8/12) in Group I yielded titers of less than 50, whereas seven of the nine patients in Group II had titers of 500 or more. In casting about for an explanation of this difference, the possibility was raised of an inadvertent selection of patients. Their clinical records were therefore reexamined. It was discovered that Group I comprised four paralytic patients, four non-paralytic, and four with mild transitory paralysis or weakness. On the other hand the patients in Group II—those with higher titers—were almost all paralytic cases. Moreover, three of the four highest titers in Group I were obtained from paralytics, whereas the two lowest values in Group II were obtained from a non-paralytic and a patient with transitory weakness. This is shown to better advantage in Table II in which the cases are regrouped according to clinical manifestations. The criteria for clinical classification were as follows: *Paralytic* patients were those who had severe or moderately severe paralysis usually with residual damage persisting at the time of discharge or transfer. Patients considered to have *mild transitory paralysis or weakness* improved rapidly and were discharged

TABLE II *

Titer of poliomyelitis virus in stools of patients in first week of illness according to clinical manifestations

Paralytic		Mild transitory weakness		Non-paralytic	
Patient	Titer	Patient	Titer	Patient	Titer
Rom	20,000	Mol	40	Biv	4000
Apo	16,000	Kur	30	Mag	16
Bra	10,000	Moo	16	Lis	4
Cav	4,000	Kun	10	Mar	4
DeA	3,000	Sta	4	Bow	4
Cas	2,000				
Con	500				
Sap	500				
Win	500				
Mun	400				
Nix	5				
Geometric mean	1308.0		15.03		21.01

* Figures similar to those in Table I.

in three to nine weeks without handicapping residual damage. *Non-paralytic* patients had characteristic spinal fluid changes but no detectable weakness at any time. Table II shows that the titers yielded by the A stools of paralytic patients were generally higher than those with less severe manifestations and vice versa. There was one exception in each group (patients *Nix* and *Biv*).

As shown in Table II, the geometric mean of the titers of the paralytic group was 1308; of the mild transitory weakness group, 15; and of the non-paralytic group, 21. Analysis of the variance performed on the log titers showed a highly significant difference between these means (P only slightly greater than 0.001). Because the data do not fall into a normal distribution curve, the appropriateness of this analysis might be questioned. Therefore, a chi-square contingency test was applied. The cases in each of the three groups were classified as either above or below the median titer (400) of the 21 subjects. Chi-square was 13.20 which for 2 degrees of freedom yields a P value again only slightly greater than 0.001.

In spite of the small samples there can be no reasonable doubt that something more than chance was responsible for the differences. Since there is no adequate indication of a difference in titer between the non-paralytic patients and those with mild transitory weakness, the chief distinction is between the latter group and those classified as paralytic. When these two were compared, again with reference to the median, in a fourfold table by Fisher's "exact" method, the difference was found to be highly significant (P from Mainland's tables [11] being less than 0.01).

Some of the stools were titrated also in roller tube cultures of monkey testicular tissue (3–7). A comparison of results in tissue culture and in monkeys (Table III) shows good agreement for results in the first week, or A stools. On the other hand, four of the B specimens (Bra, Biv, Moo, Rom), obtained in the second week of illness, show a hundred-fold difference or more in favor of tissue culture; a smaller difference (approximately 10-fold) was found in two others (Cav and Con). In the remaining five there was little or no difference in titer.

TABLE III *

Poliomyelitis virus in patients' stools titrated in monkeys and in tissue culture

Patient	1st week stool (A)		2nd week stool (B)	
	Monkey†	Tissue culture‡	Monkey	Tissue culture
Bra	10,000	3,000	70	16,000
Biv	4,000	30,000	5	1,000
Moo	16	<10	4	500
Rom	20,000	16,000	300	30,000
Cav	4,000	10,000	1,000	30,000
Con	500	1,600	150	1,000
Apo	16,000	10,000	250	1,000
DeA	3,000	5,000	3,000	10,000
Win	500	1,600	50	100
Cas	2,000	3,000	4,000	1,600
Mun	400	500	0	<10

* Figures similar to those in Table I.
† Six monkeys inoculated intracerebrally with 1 ml. of each dilution.
‡ Three roller tubes inoculated with 1 ml. of each dilution.

DISCUSSION

The fact that stools have been shown in this study to yield titers of poliomyelitis virus as high as 20,000 is in itself of more than passing interest. There are few titrations of virus in stool reported in the literature. Pearson and Rendtorff mention the occurrence of paralysis in a monkey produced by a 10^{-5} dilution of stool excreted by a child with inapparent poliomyelitis (12). Melnick titrated two pools of feces from 11 patients in the first week of illness. One pool yielded a titer of 30, the other 100 (13).

When an apparent difference in titer of virus was observed in our two groups of patients whose stools were collected in the same stage of illness in the same epidemic, a reinvestigation of clinical data pertaining to their illness was an important step in seeking an explanation. We discovered to our surprise that high titers were obtained for the most part from patients who had severe to moderately severe paralysis with residual damage. Conversely, less virus seemed to be excreted by non-paralytic patients and by those with mild transitory paralysis or weakness. Before speculating on its possible significance, it should be emphasized that this finding was an unexpected by-product of another investigation (1). As such it suffers from not being properly controlled in certain respects. For example, the stools of patients in Group I were kept frozen for a shorter period than those in Group II; although the stools of both were processed and inoculated into monkeys by the same method described above and in

the same laboratory, the procedures were carried out in different years and by different hands. Although it has not been possible to retitrate in monkeys the stools of Group I to check the earlier results, titrations in tissue culture have been performed in four (Biv, Moo, Win, and Mun): Table III shows that the A stool titers achieved in tissue culture recently are not much different from those obtained in monkeys two to three years ago. Although it is difficult to see how the factors of storage and processing could explain the observed differences in titer between the paralytic and non-paralytic groups, until the work is repeated under conditions that are better controlled, we think that the findings should be interpreted with reserve.

A comparison of virus titers obtained in monkeys and in tissue culture showed an interesting difference in the second week (B) specimens. Although there was little or no difference in titers of the A stools done by the two different methods, a considerable difference was found in a number of the B stools. The titer in tissue culture was higher by 100-fold or more than the titer in monkeys. The explanation of this is not clear. In any event, it suggests that if one is seeking variants of poliomyelitis virus with reduced pathogenicity for primates, stool passed after the first week of illness may offer a better source than earlier excretions.

SUMMARY

1. A quantitative study of poliomyelitis virus was carried out in stools obtained in the first and second weeks of illness of 21 patients during the 1949 poliomyelitis epidemic in New York City.

2. Virus in stool was titrated in monkeys and in roller tubes containing monkey testicular tissue.

3. High titers of virus, ranging from 2,000 to 20,000, were obtained in one third of the patients' stools tested in monkeys.

4. Stools of the 11 paralytic patients generally yielded high titers; stools of five non-paralytics and of five patients with mild transitory paralysis or weakness generally yielded low titers of virus.

5. A comparison of values obtained by titration in monkeys and in tissue culture showed good agreement in the first week stools. Some of the second week stools, however, showed a lower pathogenicity for monkeys while exhibiting persistently high titers for tissue culture.

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THE EFFECT OF ADRENAL STEROIDS ON AEROBIC LACTIC ACID FORMATION IN HUMAN LEUKOCYTES [1]

By SAMUEL P. MARTIN,[2] S. N. CHAUDHURI,[3] R. GREEN, AND GORDON R. McKINNEY [4]

(From the Departments of Medicine, Bacteriology, and Pharmacology, Duke University School of Medicine, Durham, N. C.)

(Submitted for publication March 23, 1953; accepted November 12, 1953)

Cortisone has an influence on the course of infectious diseases, as well as on the intensity of the inflammatory response (1–3). One of the important cells in the antibacterial defense and in the inflammatory response is the polymorphonuclear leukocyte. This cell has a very high production of lactic acid in the presence of oxygen and this rate is not altered significantly by the concentration of oxygen (4, 5). The early chemical changes in an area of inflammation such as lowering of pH are in part due to the aerobic lactic acid production by leukocytes.

The present study was undertaken to determine the effect of active and inactive adrenal steroids on this metabolic characteristic of human leukocytes *in vitro*. Compound E and F were found to inhibit aerobic lactic acid production.

MATERIAL AND METHODS

The method for collection and preparation of blood has been previously described (4). Healthy donors in the fasting state were used. The blood was collected from the cubital veins into a non-wetting system. All glassware was siliconed (G.E. Silicone SC 77). Heparin (0.04 mg. per ml.) or a cation exchange resin (Dowex-50) was used to prevent coagulation. The leukocytes were concentrated by the method previously described (4) in which dextran (intrinsic viscosity 0.46) is added to produce a final concentration of 1 per cent. The dextran induced rouleaux formation and rapid sedimentation of red blood cells. After removal of the supernatant plasma containing the leukocytes, the cells were concentrated by centrifugation at 50 x G for 15 minutes. The plasma was decanted and the white cells were resuspended in a sufficient volume of buffered saline (4) with 100 mg. per cent glucose to obtain a concentration of 5,000 to 25,000 cells per cu.mm. Cortisone and hydrocortisone were dissolved in concentrations of 0.01 to 10.0 micrograms per ml. Cholesterol, desoxycorticosterone and Reichstein's compound S [5] in final concentration of 10 to 15 micrograms per ml. were also used. The suspension of leukocytes in buffered saline, with and without the steroids, was distributed into 25 ml. siliconed Erlenmeyer flasks which were suspended in a constant water bath at 37° C. and shaken gently in the horizontal axis at the rate of 10 oscillations per minute. Lactic acid was measured by the technique of Barker and Summerson (6). Measurements of initial lactic acid were made before the cells were placed in the water bath and after four- and six-hour periods of incubation. The values are reported as micromoles of lactate produced by 10,000,000 cells per hour.

In one group of subjects, the *in vitro* effect of cholesterol, cortisone, hydrocortisone, desoxycorticosterone, and Reichstein's compound S on lactic acid production was measured. In another group, the effect of cortisone on the production of lactate and the utilization of glucose, oxygen, and glycogen was measured by techniques previously described (4).

RESULTS

The effect of the various steroids on aerobic production of lactic acid by leukocytes from untreated subjects is presented in Table I. Cortisone and hydrocortisone caused a diminution, while compound S an increase. Cholesterol had no statistically significant effect at four hours. With hydrocortisone, the change was manifest at four hours and was still evident at six hours. With cortisone, the effect at four hours was slight ($P = < 0.05$) and more marked at six hours. Compound S produced a significant increase in lactate at four and six hours, while desoxycorticosterone produced a rise at six hours which was of questionable significance ($P = < 0.05$). This was probably due to the limited number of observations. These experiments were carried out with a concentration of 10 micrograms per ml. of steroid. Experiments on lactate production by

[1] This work was supported in part by a Research Grant from the National Institutes of Health, Public Health Service, the Baxter Laboratory, and the Spanel Foundation.

[2] Markle Scholar of Medicine.

[3] Permanent Address: Indian Institute for Medical Research, Calcutta, India.

[4] Fellow of the American Cancer Society.

[5] Obtained through the courtesy of Dr. Augustus Gibson of Merck and Co., Rahway, New Jersey, and Dr. William Haines, The Upjohn Co., Kalamazoo, Michigan.

TABLE I

The effect of adrenal steroids on aerobic lactate formation *

	No. of subjects	0–4 hr. change	Change	0–6 hr. change	Change
Control	30	0.59 ± 0.04		0.62 ± 0.05	
Desoxycorticosterone	8	0.64 ± 0.09	+0.05 ± 0.02	0.75 ± 0.05	+0.1 ± 0.02
Cortisone	30	0.53 ± 0.09	−0.06 ± 0.03	0.48 ± 0.04	−0.18 ± 0.02*
Hydrocortisone	16	0.46 ± 0.03	−0.10 ± 0.03†	0.51 ± 0.04	−0.10 ± 0.02*
Compound S	8	0.68 ± 0.05	+0.13 ± 0.05†	0.68 ± 0.04	+0.06 ± 0.02
Cholesterol	8	0.62 ± 0.04	0.02 ± 0.04		

* Values are expressed in micromoles of lactic acid per 10,000,000 cells per hour ± S.E.
† Indicates probability of >0.01.

leukocytes exposed to various concentrations of hydrocortisone are presented in Table II. There was no significant change with 0.1 microgram per ml. This would indicate the minimal effective concentration was between 0.1 and 1.0 microgram per ml. In four trials with broken cells no effect could be noted.

The data on the effect of cortisone on lactate production, and on glucose, glycogen and oxygen utilizations are presented in Table III. There was a diminution of lactate production at four and six hours. The former was of questionable significance and the latter was highly significant. The utilization of glucose was lowered at four and six hours. There was no significant change in oxygen and glycogen utilization. Disruption of the cells by grinding with alumina gel blocked the effect of cortisone and hydrocortisone.

DISCUSSION

These data indicate that some of the adrenal steroids have a direct effect on human leukocytes *in vitro*. The effect of cortisone was diminution in aerobic lactate production and in glucose utilization. The effect was seen only in intact cells which had been handled with care to prevent injury of cell surfaces. The cellular site of action of the steroids in their effect on lactate production is unknown. Preliminary data would indicate that part of the lactate may be produced through an enzymatic mechanism other than lactic dehydrogenase. This interpretation is based on the lack of complete inhibition of lactate production by oxamic acid. Previous work by Hakala, Glaid, and Schwert (7) has shown oxamic acid to be a specific inhibitor of lactic dehydrogenase. With final concentrations as high as 0.02 M there is less than 40 per cent inhibition. Leukocytes show a high glyoxylase activity and this may account for the remainder of the lactic acid. Glutathione in excess (0.005 M) markedly diminishes lactic acid production (8, 9). Since lactic dehydrogenase would seem to account for only a part of the lactate produced by the polymorphonuclear leukocyte, the alteration induced by cortisone could arise from either its effect on pyruvate production or at another site, such as glucose absorption or utilization. Previous work has indicated a close correlation between glucose utilization, glucose concentration, and lactate production by the leukocyte (10). Since the hormones have no effect on broken cells, it would seem that the mode of action is related to the integrative behavior of the cell rather than to the mechanisms of terminal respiration.

The lack of alteration in oxygen utilization despite changes in lactate production cannot be accounted for in the present experiments. Previous studies with human leukocytes have shown

TABLE II

The effect of varying quantities of hydrocortisone on lactate production by human leukocytes in vitro *

Concentration of hydrocortisone *Micrograms/ml.*	5	1	0.1
Change *micromoles/ 10 million cells/hr.*	−0.08 ± 0.03	−0.08 ± 0.02	−.004 ± 0.02

* Studies were carried out at 4 hours. Values are reported as micromoles change over control. Values represent ten subjects in each determination.

TABLE III

The effect of cortisone on metabolism of human leukocytes expressed as micromoles per 10 million cells per hour

	No. of subjects	Control		Compound E	
		0–4 Hr.	0–6 Hr.	0–4 Hr.	0–6 Hr.
Lactic acid	30	+0.59 ± 0.04	+0.62 ± 0.05	+0.53 ± 0.09	+0.48 ± 0.04*
Glucose	30	−0.205 ± 0.02	−0.106 ± 0.02	−0.150 ± 0.017*	−0.070 ± 0.016*
Oxygen	7	−0.178 ± 0.04	−0.139 ± 0.030	−0.168 ± 0.03	−0.127 ± 0.03
Glycogen	5		−0.007		−0.007

* Indicates probability of >0.01
10 Micrograms of Compound E per ml.

a reciprocal relationship between lactate production and glucose utilization on the addition of glucose or on cell injury. The present experiments may indicate a site of action of the hormone not related to the factors controlling oxygen uptake.

The effective levels in these experiments were found to lie between 1.00 and 0.1 micrograms per ml. Nelson, Sandberg, Palmer, and Tyler (11) found that 200 mg. of compound F given orally produced a blood level of 0.60 micrograms per ml. This level was associated with marked change in polymorphonuclear cell count. This is in the same range as the present experiments.

The effect of compound S is the same as the response noted on injury (4). It may be a pharmacological effect or may be due to some factor present in the material such as pyrogen contamination (12). Selye states that desoxycorticosterone and compound S have a prophlogistic effect (13).

The relation of the antiphlogistic and metabolic effect of cortisone and hydrocortisone is not known. The former has been attributed to the action of these agents on the blood vessels (14). The metabolic effects noted here may also be related to the anti-inflammatory action. This alteration in the leukocyte metabolism would result in a slowing of lactic acid production, less change in pH in the area of inflammation, and possibly longer survival of cells. These factors could be manifested by a slow and abnormal evolution of the inflammatory reaction.

SUMMARY

Cortisone and hydrocortisone have a direct effect on human leukocytes. These hormones cause diminution in production of lactic acid. Oxygen utilization is not altered. It is postulated that these metabolic alterations may play a role in the antiphlogistic effects of adrenal steroids.

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EXPERIMENTAL IMMERSION FOOT. I. THE EFFECTS OF PROLONGED EXPOSURE TO WATER AT 3° C. ON THE OXYGEN TENSION AND TEMPERATURE OF THE RABBIT LEG [1]

By HUGH MONTGOMERY, ORVILLE HORWITZ, GEORGE PEIRCE, AND ANN SAYEN

(From the Vascular Section of the Robinette Foundation, Medical Clinic, Hospital of the University of Pennsylvania, Philadelphia, Pa.)

(Submitted for publication March 16, 1953; accepted November 25, 1953)

Prolonged exposure of a limb to cold water causes the disease known as immersion foot. The widespread literature has been reviewed elsewhere (1). Neuromuscular dysfunction and histopathological changes in nerve, muscle, and other tissues are readily demonstrated after such exposure. The question of how cold short of freezing damages the tissues has not been settled. Goldschmidt and Light (2) and Lewis and Love (3) suggested that the poor dissociation of oxygen from cold hemoglobin may make the tissues anoxic. It is true that the supply of oxygen by cold hemoglobin is meager (4) but it is also true that the utilization of oxygen by tissues is conspicuously lowered at such reduced temperature (5). We have demonstrated in short term experiments a reduced oxygen tension of the skin of man exposed to cold (6). The present experiments were carried out in order to learn to what extent, if any, prolonged exposure to cold lowers the oxygen tension of muscle and of the subcutaneous space, and whether oxygen inhalation will increase the oxygen tensions. Preliminary experiments on the effect of cold on the skin of human extremities were abandoned because of the danger involved. Rabbits were chosen because there has been some experience in producing immersion foot in that animal (7). Parallel studies were made of the temperatures of the muscle and of the subcutaneous tissue in order to learn the degree of chilling of tissues and in order to get some idea of the rate of blood flow through the tissues during prolonged chilling.

[1] This investigation was supported by research grant 392 (C3) from the National Heart Institute, of the National Institutes of Health, Public Health Service, and by a research contract between the University of Pennsylvania and the Office of Naval Research.

METHOD

The open tip platinum electrode (8) for estimating oxygen tension was utilized as previously described (6) except for several modifications. The electrode consists of the sharpened tip of a 0.2 mm. platinum wire, the rest of the wire electrically insulated. At voltage 0.6V. the current measured is linearly proportional to the rate of diffusion of oxygen to the electrode. For greater sensitivity to lower oxygen tension in these studies, electronic amplification was substituted for the galvanometer used in previous studies. The circuit was a modification of that used by Moody for ionization chamber work (9). The electrodes and their wire connections were insulated to minimize electrical leakage in the special condition of underwater work. In spite of this, electrical leakage was not abolished in all experiments. When it occurred the data had to be discarded as were data from an occasional unresponsive electrode. The electrodes were placed in subcutaneous areas rather than in skin because rabbit skin is so thin as perhaps to allow its oxygen tension to be influenced by oxygen of the surrounding water and of the subcutaneous space. All data were corrected for direct temperature effect on electrical current as previously found for these electrodes (6). Measurements were made without calibrating (6) the electrodes, and the data are presented as relative (Figure 3) since the absolute values of oxygen tension are not known. We were especially interested in the changes in oxygen tension in muscle because of a parallel study we made of the neuromuscular dysfunction and histopathological changes resulting in the muscles of the legs of another series of rabbits similarly exposed (10).

Copper-constantan thermocouples were used in conjunction with the Brown potentiometer to record all temperatures at three-minute intervals.

The apparatus for holding the rabbit and exposing the left hind limb to cold water was a modification of that used by Lange, Weiner, and Boyd (7). Each rabbit-holding stall was constructed in the following manner. A vertical plexiglass tube having a wall thickness of one-eighth inch, an outer diameter of three inches, and a length of fourteen inches was fitted flush up into a hole in a horizontally held board having a thickness of five-eighths inch. The plexiglass tube was furnished with a water inlet at its lower end and an outlet tube near its

TABLE I

Average temperatures, and fluctuations of temperatures, of water, of muscle, and of subcutaneous space within the following time periods *

Rabbit No.		Temperature °C. 0–1 hrs.	1–6 hrs.	6–12 hrs.	12–24 hrs.	24–36 hrs.	36–48 hrs.	48–60 hrs.	60–72 hrs.
	Water	2	2	2	2	2	2	2	2
1	Muscle		8±2	6	5	5	6	7±1	5±1
	Subcut.		6±1	6	4±1	3	4±1	4	3
	Water	3	3	3	4±1	3	2	2	2
2	Muscle		9±3	8	7±1	5±1	5±1	4+1	4±1
	Subcut.		4	4	4±1	3	3	3	3
	Water	1	2±1	2	1	1	2±1	1	2±1
3	Muscle		3±1	3±1	2	2	2±1	1	2±1
	Subcut.		3±1	2	2	1	2±1	1	2±1
	Water	2	3	3	3	3	2	2	
4	Muscle		6	5	4±1	4±1	2	2	
	Subcut.		4	3	3	3	2	2	
	Water	2	3	3	3	3	2	2	
5	Muscle		5±1	4±1	5±1	5±1	4	3±1	
	Subcut.		4	3	3	4±1	3	3	
	Water		3±1	2	2	2	3±1	2	
6	Muscle		6±1	7±1	6±2	12±8	10±6	5	
	Subcut.		4±1	3	3	5±3	5±3	2	
	Water	2	3	3	3	2	2	2	2
Approx. averages	Muscle		6	6	5	6	5	4	3
	Subcut.		4	4	3	3	3	3	3

* Six rabbits. Temperatures °C. are shown of the water to which each rabbit's left hind leg was exposed, of the muscle of the exposed leg, and of the neighboring subcutaneous space. The temperatures throughout each time period are analyzed in detail. Temperatures tabulated to nearest 1°C. Fluctuations less than ±1°C. not tabulated. Fluctuations of ±1°C. to ±1.4°C. as ±1°C., etc.

upper end, just below the board. A heavy-duty electrically driven centrifugal pump was inserted in a half-inch rubber tubing connecting a large-volume thermostatically controlled water cooling tank with each inflow tube. Starting the pump raised the water through the vertical tube, and the water overflowed through the outlet tube back into the cooling tank. This served to maintain a water surface level well above the knee of the left hind leg. The water in the vertical tube could be withdrawn, and be replaced by air, by turning off the pump.

The rabbit was placed on the board with the left hind leg in the vertical tube, the right hind leg in a similar tube filled with air, and the body gently held by straps. To complete each stall there was a nearly airtight housing over the board for use when the temperature of the air surrounding the rabbit was decreased, or when oxygen was administered.

Air temperature could be decreased by pumping cold air, from above the surface of the water in the cooling tank, into the stall. When oxygen was used it was administered from an oxygen tank by flow at 10 liters per minute directly into the stall. This rate of flow, with a rabbit in the stall, presented a 640 to 760 mm. oxygen tension as measured by a calibrated Pauling meter.

PROCEDURE

The experimental animals were divided into two groups. Numbers 1 through 8 were used to study changes in temperature of muscle and of the nearby subcutaneous area in response to exposure of a limb to cool water (averaging 2.3° C.). In the case of rabbits numbers 1 through 6 the exposure was continuous, in numbers 7 and 8 intermittent. Numbers 9 through 13 were used to study the changes in oxygen tension under the same conditions.

Female rabbits averaging 3 kilograms in weight were selected in order to fit the rather exacting dimensions of the stalls. Throughout the experiment they were fed a desiccated rabbit ration and water ad lib. The left hind leg was depilated two days before using the rabbit in an experiment in order to allow any inflammatory response to subside. With the exception of rabbit No. 12, none of the limbs had been previously exposed to cold.

The thermostat of the water cooling device remained at the same setting throughout all experiments.

Thermocouples and electrodes were inserted under ether-atropine anesthesia, except in rabbit No. 12, in which the necessary muscle dissection was performed under the narcotizing effect of two days of cold exposure.

In the first eight rabbits thermocouples were tied deep in the extensor group of muscles half-way between the

TABLE II

Ranges of air temperatures and of body temperatures of same rabbits as shown in Table I *

Rabbit No.	Maximum and minimum temperatures °C.									
		Body								
	Air	0–1 hrs.	1–6 hrs.	6–12 hrs.	12–24 hrs.	24–36 hrs.	36–48 hrs.	48–60 hrs.	60–72 hrs.	Mean
1	22.0	36.8	36.8	38.3	38.5	38.5	39.0	39.2	38.8	38.2
	26.0	36.8	38.2	38.7	37.4	37.4	38.7	38.3	38.1	
2	13.0	36.8	37.5	38.5	37.7	38.8	38.5	38.4	38.5	38.2
	17.0	37.5	38.5	37.5	38.9	38.2	37.8	39.0	39.5	
3	12.0		37.5	38.0	37.0	37.3	37.0	37.0	37.3	37.3
	16.0		38.0	37.3	37.8	37.5	38.0	37.3	36.5	
4	16.0	37.0	37.0	37.7	37.5	37.9	38.0	37.5		37.8
	18.0	37.0	37.7	37.7	37.9	38.3	38.6	39.5		
5	24.0	37.2	37.2	38.0	38.0	38.0	38.2	38.7		37.9
	26.0	37.2	38.0	38.0	38.0	37.2	38.5	38.3		
6	21.0	35.2	36.0	37.9	37.8	38.7	38.6			37.6
	26.0	36.0	38.0	38.1	38.8	37.8	38.0			

* Six rabbits (same animals as in Table I). Body temperatures obtained by thermocouple under belly, skin upon dry wood floor. Extremes of body temperature tabulated for each time period.

knee and the ankle of the left leg and in the nearby subcutaneous space. Interference with blood supply was avoided as far as possible when placing ligatures. After the rabbit was placed in the holding stall, body temperature was recorded from a thermocouple that had been inserted into the subcutaneous space of the belly wall, the skin of which presented against the dry wood floor of the stall. A fourth thermocouple recorded the temperature of the water (or air) surrounding the left hind leg. A fifth recorded air temperature. In this group of animals left hind legs were exposed continuously to cold for from 66 to 130 hours.

In the second series (rabbits 9 through 13) platinum electrodes for the measurement of oxygen tension were inserted. Simultaneous measurements of oxygen tension and of limb temperature were avoided because of the complexity of such experiments and in order to minimize current leakage through water between the two electrical systems. In most instances only one electrode was tied into the muscle, and one in the nearby subcutaneous space, in the left hind leg. In rabbit No. 11 two electrodes were used subcutaneously.

As soon as the electrodes were in place, the rabbit was placed in its stall. All rabbits except No. 12 were recovering gradually from the anesthesia, and their left hind legs were at first in an air-filled tube. The duration of experiments ranged from two hours to ten hours and forty-five minutes. In general, the first exposure of a rabbit leg was to air at room temperature, and the rabbit was given air, oxygen, and air to inhale. Then cold water was allowed to flow around the leg, and the rabbit again inhaled the gases in the same order. Some experiments were carried further, as shown in Table III and in Figure 3.

Unlike the others, the leg of rabbit No. 12 was studied after chilling for two days, and again was studied on the third day. The electrodes remained in place between the two periods of study. A comparison of the results of these studies on successive days, and observations with paired electrodes (rabbits No. 11 and No. 13) afford an understanding of the variability of oxygen tension data obtained in the difficult condition of underwater electrical work (Figure 3).

In the oxygen tension experiments occasional measurements of the temperature of the water (average 2° C.), and of the surrounding air (average 25° C.) demonstrated no regular difference from these conditions in the first experimental group.

RESULTS

During the prolonged experimental period the rabbits consumed food and water, and eliminated urine and feces normally. The rabbits remained alert, and appeared healthy throughout all experiments. On removal of the thermocouples and electrodes the tissues were found to be clean and grossly uninfected.

A. Changes in temperature of muscle and of the nearby subcutaneous area in response to exposure to cool water

Eight rabbits (No. 1 through No. 8) were studied. The temperature of the water averaged 2.3° C., and fluctuated less than 1° C. except as

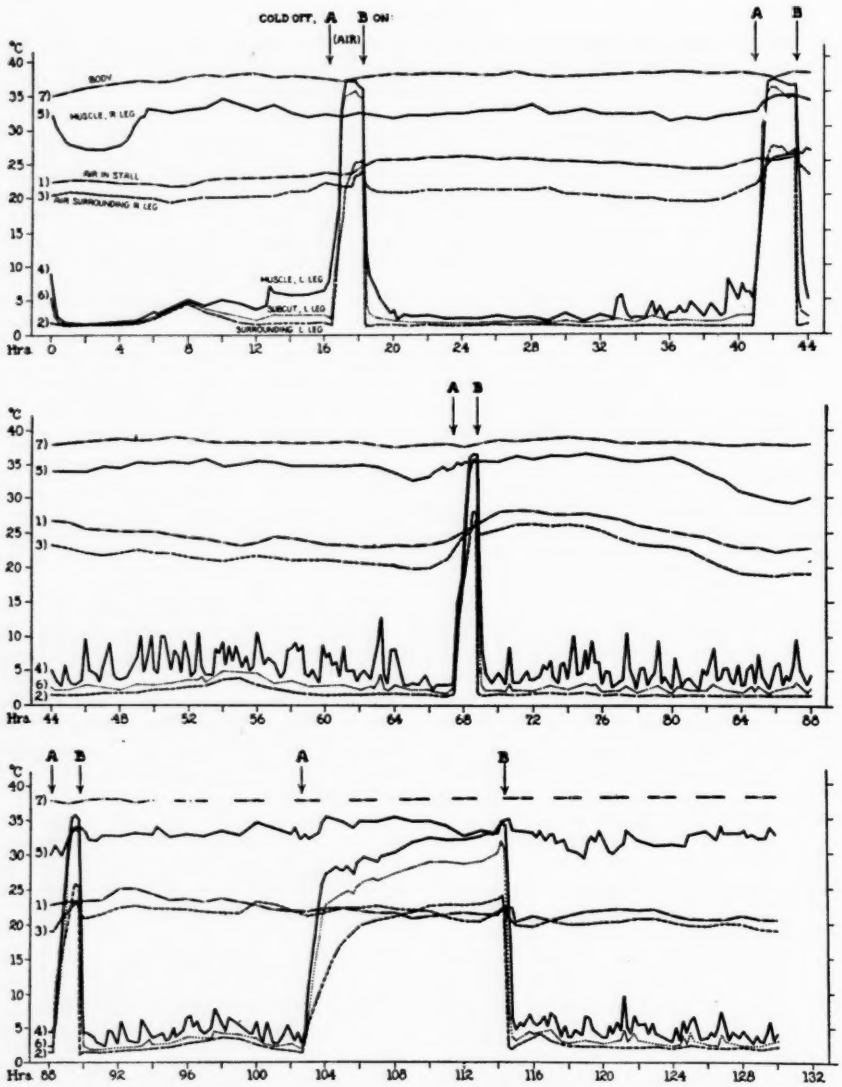
COLD OFF. A B ON
(AIR)
A B
°C
BODY
MUSCLE, R LEG
AIR IN STALL
AIR SURROUNDING R LEG
MUSCLE, L LEG
SUBCUT. L LEG
SURROUNDING L LEG
Hrs. 0 4 8 12 16 20 24 28 32 36 40 44
Hrs. 44 48 52 56 60 64 68 72 76 80 84 88
Hrs. 88 92 96 100 104 108 112 116 120 124 128 132

FIG. 1. FLOW FLUCTUATIONS AND RECOVERIES OF BLOOD FLOW AFTER WITHDRAWAL OF LEG FROM COLD WATER

Rabbit No. 7. Left hind leg in cool water for 130 hours except for temporary quick withdrawal of the water at 16 hours, 41 hours, 67 hours, 88 hours, and slow withdrawal at 102 to 114 hours. Right hind leg in air. Time in hours shown in abscissae, temperature in °C. in ordinates. Graph shows temperatures of the following:

1 = T °C. of the air surrounding the rabbit.
2 = T °C. of the water (or air) surrounding the exposed (left) leg.
3 = T °C. of the air surrounding the unexposed (right) leg.
4 = T °C. of the muscle of the exposed (left) leg.
5 = T °C. of the muscle of the unexposed (right) leg.
6 = T °C. of the subcutaneous area of the exposed (left) leg.
7 = T °C. of the body (subcutaneous, belly against dry wood).

shown in Table I. Air temperatures and body temperatures are given in Table II. The visible responses of the leg to cold were much as reported by Lange, Weiner, and Boyd (7). The leg became pink and remained so throughout the experiment. Muscle temperature decreased rapidly, and even more profoundly than in their experiments (Table I). Subcutaneous temperatures averaged a little lower than muscle temperature. After twenty-four hours there was considerable swelling of the exposed leg and some edema of the contralateral, unexposed, equally dependent leg. In subsequent days edema, predominating in the exposed leg, became severe. Occasional motions of the exposed leg appeared to result solely from the action of muscles which were above the level of the cold water. Muscle action caused transient increases of temperature of the submerged muscle. Since the animals were sacrificed at the termination of each experiment, and recovery following withdrawal from cold was not studied, the usual rapid loss of edema, and the persistent neuromuscular dysfunction, were not observed. Fluctuations in temperature of muscle and of the neighboring subcutaneous spaces were slight, with the exceptions of rabbit No. 6 (Table I) and rabbit No. 7 (Figure 1).

The data from rabbit No. 7 (Figure 1) are not tabulated with the first six rabbits. As in other experiments the left hind leg of rabbit No. 7 was exposed to the usual cold water but unlike the other experiments the cold water was withdrawn from the leg, and air was substituted for the water each day for a sufficient time to allow the temperatures of the leg to rise to a steady value. In general these temperatures rose to levels close to body temperature, and there was no day-to-day decrease in this effect. Figure 1 shows the extent of this recovery of limb temperature, and therefore of blood flow after various times of exposure to cold water. The average recovery of muscle temperature was to 36° C., and of subcutaneous space to 35° C. The values can be compared with those in the right (unexposed) leg of five of the rabbits in this series, the average muscle temperature of which was 35° C. (11 determinations) and subcutaneous temperatures 34° C. (11 determinations). During leg cooling, rabbit No. 7 most clearly illustrated a conspicuous rhythmic variation in leg temperature, suggesting the "hunting reaction" described for human skin by Lewis (11) and for human muscle by other investigators (12). The fluctuation in temperature in the muscle of this rabbit's leg, at times amounting to 5° C., must represent great increases of blood flow above the relatively low flows in the intervals between the fluctuations. The leg of a last rabbit, No. 8, was exposed in the interrupted way in which the leg of No. 7 was exposed. It failed to show the temperature fluctuations shown by rabbits No. 6 and No. 7, but until the termination of the experiment at one hundred hours, the temperature of the muscle and of the nearby subcutaneous space recovered, when the water was withdrawn, to values near body temperature. In the case of these two rabbits, the circulation of the limbs recovered after prolonged, though interrupted chilling.

An attempt was made to determine whether these temperature fluctuations were truly "spontaneous" and therefore of the nature of the "hunting reaction," or whether they were induced by motion of the leg. Four rabbits were studied. The leg-cooling chamber was made larger in order to allow enough freedom of motion for recording. Legs were cooled for several days and leg motion was continuously recorded simultaneously with temperature. All fluctuations of muscle temperature greater than 1° C. were found to correspond to muscular movement. Motion elicited by faradic stimulation of the skin over the left sciatic notch also caused such increments in muscle temperature. The fact that an arterial tourniquet prevented these muscle temperature rises but did not prevent leg motion indicates that the temperature increases were almost entirely a result

A = cold water withdrawn from around left hind leg.
B = cold water replaced around left hind leg.

All readings of temperatures were made at 3-minute intervals and were begun within 5 minutes after starting cold water exposure to the left leg. At 94 hours the thermocouple recording body temperature was broken, and occasional body temperatures were taken by inserting a mercury thermometer into the rectum.

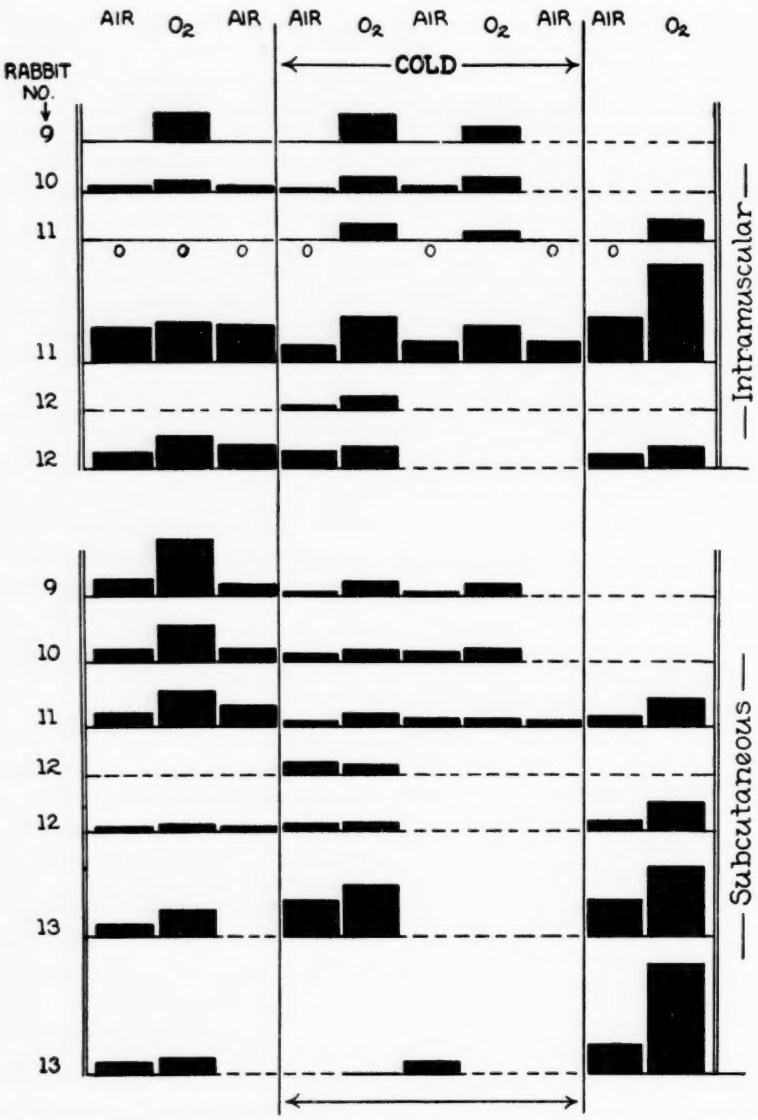

FIG. 3. CHANGES IN OXYGEN TENSION OF MUSCLE AND OF NEARBY SUBCUTANEOUS SPACE OF THE LEFT HIND LIMB OF THE RABBIT

The heights of the blocks vary directly with oxygen tension (see Text).

"Air" denotes air inhalation, "O_2" oxygen inhalation. The results of immersion of the limb in 2° C. water are shown under "COLD," and the times of exposure are shown in Table III. The dotted lines indicate incomplete studies. No data of an electrode were discarded prior to the termination of any experiment, with the exception of those of rabbit No. 9, discontinued early because the galvanometric readings repeatedly swung erratically to zero. A second muscle electrode in the experiment with rabbit No. 10 was completely unresponsive and the data are not included. Rabbit No. 12 was studied on two successive days. Rabbit No. 13 had two subcutaneous electrodes.

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EXPERIMENTAL IMMERSION FOOT. II. FUNCTIONAL AND HISTOLOGICAL CHANGES IN THE RABBIT LEG EXPOSED TO WATER AT 3°C., AND THERAPEUTIC TRIAL OF CORTISONE AND OF INHALED OXYGEN [1]

By ORVILLE HORWITZ, HUGH MONTGOMERY, ANN SAYEN, AND HERBERT MESCON

(From the Vascular Section of the Robinette Foundation, Medical Clinic and from the Department of Dermatology, Hospital of the University of Pennsylvania, Philadelphia, Pa.)

(Submitted for publication March 16, 1953; accepted November 25, 1953)

Prolonged immersion of the leg of a rabbit in cold water produced pathological and functional changes (1, 2) including muscle fibrosis. The cold rather than the water is responsible (1, 2).

The oxygen tension of the muscle of the rabbit's leg is usually reduced during the immersion of the leg in water at a temperature of 3°C., and inhalation of high concentrations of oxygen increases the oxygen tension to or above the pre-immersion level (3). Thinking that this might be beneficial, and that by the administration of cortisone lessening of fibrosis or some other advantage might be gained, this series of experiments was designed to test separately the effects of oxygen and of cortisone upon the functional and pathological changes of the rabbit leg exposed to cold water.

METHOD

The left hind legs of 107 rabbits were exposed to water at 3°C. by a modification (3) of the method used by Lange, Weiner, and Boyd (1). Female rabbits, averaging three kilograms in weight were used. The left hind legs were depilated two days before their exposure. The first 23 rabbits were fed oats only, the remainder were fed desiccated rabbit ration which appeared to be a much healthier diet. An adequate intake of food and water was maintained throughout the entire exposure period. Rabbits were weighed daily. All surviving animals were sacrificed thirty days after exposure.

Oxygen was administered as described previously (3), in such a manner that, throughout the period of immersion of the left hind leg, 25 rabbits breathed a mixture of 80 to 90 per cent oxygen. This was accomplished by allowing the oxygen to escape into the stalls at a rate of two to three liters per minute and to be recirculated through soda lime to eliminate the carbon dioxide.

Cortisone was administered to 16 rabbits by subcutaneous injection of 10 mg. daily for two weeks, starting on the first day prior to immersion of the left hind leg.

[1] This investigation was supported by research grant 499 (C3) from the National Heart Institute, of the National Institutes of Health, Public Health Service.

Functional tests were applied to both hind legs on the day prior to exposure, and on each day or each two days subsequently, to test the animal's ability to: 1. Spread the toes; 2. dorsiflex the foot; 3. hop; and 4. to bear weight. We also noted, 5, the extent of edema formation.

When a normal rabbit is held by the scruff of the neck and lowered toward the floor it spreads its toes and dorsiflexes its foot at the ankle. Loss of these functions and any tendency to drag the left hind leg while hopping was considered abnormal. Weight-bearing was tested by lowering the rabbit upon a spring scale in such a manner that the rabbit was forced to bear as much weight as possible upon the leg in question. Edema was graded as slight, moderate, or marked.

Histological studies were made of the muscle of both hind legs, regardless of the length of time each animal survived after exposure. On the 30 ± 1 day after completing exposure, all surviving animals were chloroformed. The hind legs were removed immediately after death, and placed in formalin for future study. Later a muscle specimen was removed from the extensor muscles of the foot 2 inches below the knee (3 inches below the level of immersion) for microscopic study. All staining of microscopic slides was with hemotoxylin and eosin.

Toxicity to oxygen has been reported in rabbits, featuring widespread pulmonary lesions (4). For this reason, the heart and lungs were examined in 9 of the rabbits receiving oxygen, in 2 of the rabbits receiving cortisone, and in 12 of the control series.

Because of the fact that renal lesions and glycosuria (5) have been reported in rabbits receiving cortisone, daily qualitative tests for glucose were performed on the urine of the 12 rabbits receiving this preparation. The kidneys of these rabbits were also subjected to gross and microscopic study at the time of death.

Thirteen of the 107 animals were discarded because of mechanical failure of the cooling apparatus, escape of the animal, death during exposure, or obvious systemic illness at the time of, or shortly after, removal from the stall. Of the 94 rabbits remaining, 27 died within the first 30 days after exposure. The other 67 were studied for a 30-day period by the functional tests. Pathological studies were made on the first 56 of the 94 animals.

An additional 4 rabbits were confined within the apparatus for a period of 50 hours with legs dependent but not exposed to cold water. All 4 of these control ani-

TABLE I

Effect of cortisone and oxygen inhalation on functional recovery of cold-exposed legs of 107 rabbits at end of 30-day period

Hours of exposure	Total no. (Controls and treated) studied	Expired before 30 days (excluded from study)	Rabbits included in study	No treatment		Cortisone		Oxygen†		Totals	
				Returned to normal*	Did not return to normal	Returned to normal	Did not return to normal	Returned to normal	Did not return to normal	Returned to normal	Did not return to normal
64	4	2	2	0	2	—	—	—	—	0	2
56	4	4	0	—	—	—	—	—	—	—	—
42–45	36	12	24	6	7	4	2	5	0	15	9
30–31	26	6	20	5	3	3	3	4	2	12	8
20–24	19	2	17	6	4	—	—	6	1	12	5
16	3	1	2	1	0	—	—	1	0	2	0
8	2	0	2	2	0	—	—	—	—	2	0
Totals	94‡	27	67	20	16	7	5	16	3	43	24

* Criteria for return to normal was full ability to spread exposed (left) hind toes, dorsiflex the foot, and have normal appearance of hopping. Weight bearing on left and right legs was not considered in this analysis.

† Discounting the 64-hour exposure group a significantly larger number of rabbits returned to normal statistically among those receiving oxygen than among the control (or no treatment) group. (Chi square = 4.1, $P < 5\%$.)

‡ The first 56 of these rabbits were used in the pathological study. The data on 13 rabbits of the original 107 were discarded for reasons given in "Method."

mals were subjected to the functional tests but none was included in the pathological study.

The number of rabbits used in each of the studies is given in the tables.

RESULTS

Functional

1. Ability to spread the toes of all left hind legs was lost when they were examined immediately after exposure, but was restored in an average of 5.5 days in the group which was exposed for 24 hours, of 8.2 days in the group exposed for 30 hours, and of 10.9 days in the group exposed for 45 hours.

2. Ability to dorsiflex the foot of the left hind leg was totally lost for an average of 18 days in rabbits whose legs were exposed to cold for 45 hours or more. In those exposed less than 30 hours this ability was almost invariably restored in 15 days or less. Two rabbits with leg exposure for 64 hours had not regained this ability when sacrificed 30 days later.

3. The return of a rabbit's ability to hop normally 30 days after exposure of one leg returned in inverse proportion to the duration of exposure.

4. The ability to bear weight was most impaired immediately after exposure, was much more impaired in the left (exposed) leg than in the right, and varied inversely as the time of exposure. Weight-bearing had returned to normal 30 days after exposure in 96 per cent of the rabbits' legs exposed 45 hours or less.

5. The rabbits lost about 0.5 Kg. in weight during exposure in spite of the leg edema. This weight was regained by the end of thirty days in all animals except one-third of those receiving cortisone. Marked edema was always observed in both hind legs immediately following exposure. It was more pronounced in the exposed leg, and almost invariably cleared up within forty-eight hours after exposure.

The group treated with oxygen differed in two ways. The per cent of limbs returning to normal at the end of thirty days by the criteria given (Table I) was maintained significantly higher. Second, in rabbits with legs exposed for 30 hours,

TABLE II

Days required to recover any ability to spread toes in rabbits breathing 85 per cent O_2 and air. Rabbits were exposed to water at 3°C. for varying periods of time

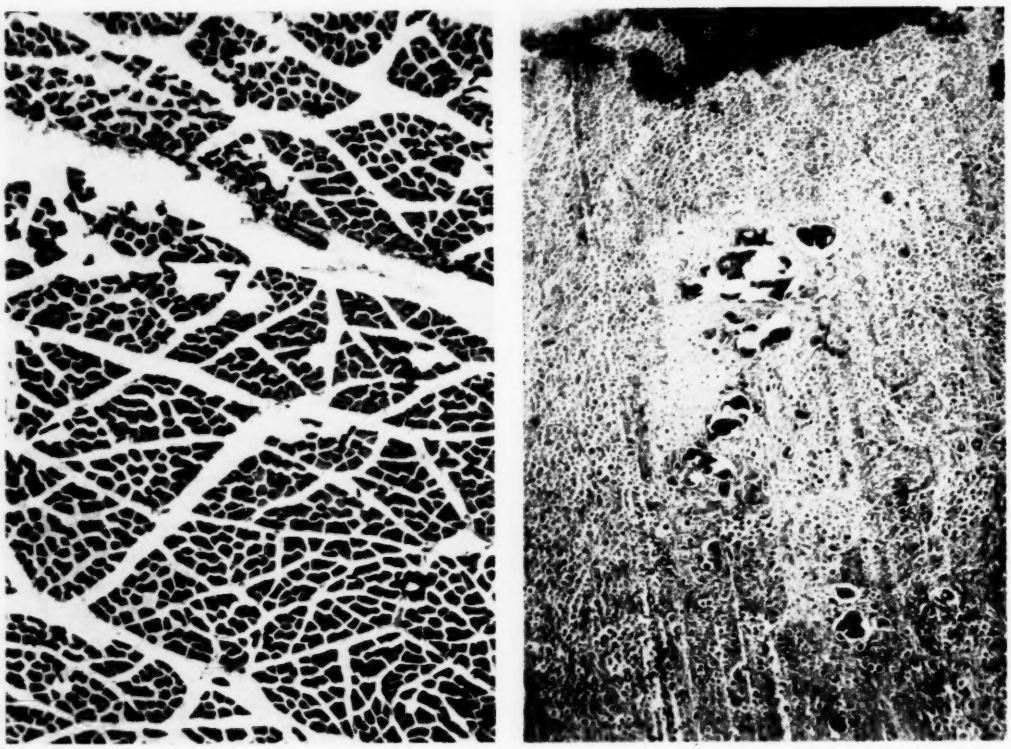
Hours of exposure	Average number of days required to regain ability to spread toes (left)		Standard deviation of average number of days		Number of rabbits	
	Control	Oxygen	Control	Oxygen	Control	Oxygen
24	7.0	4.5	1.2	1.8	8	7
30	11.0	6.5	1.0	1.3	8	6
45	12.0	11.0	2.1	1.6	13	5

TABLE III

Frequency and degree of histopathological findings in muscle

Hours exposure to cold	No. of cases	Basophilia		Cellular infiltration		Giant cells		Edema		Fragmentation	
		No. involved	Degree invlmt.*	No. involved	Degree invlmt.	No. involved	Degree invlmt.	No. involved	Degree invlmt.	No. involved	Degree invlmt.
50–60 (exp. L leg)	8	3	S	3	M	0	—	3	M	6	C
(unexp. R leg)	8	1	S	1	S	0	—	1	S	1	S
42–45 (exp. L leg)	19	7	M	8	M	0	—	7	M	13	M
(unexp. R leg)	19	2	S	2	S	0	—	2	S	2	S
30–31 (exp. L leg)	13	1	M	0	—	1	M	1	S	5	M
(unexp. R leg)	13	0	—	0	—	0	—	0	—	1	S
20–25 (exp. L leg)	16	2	S	0	—	0	—	1	S	3	M
(unexp. R leg)	16	0	—	0	—	0	—	0	—	1	S

* S = slight, M = moderate, C = considerable.

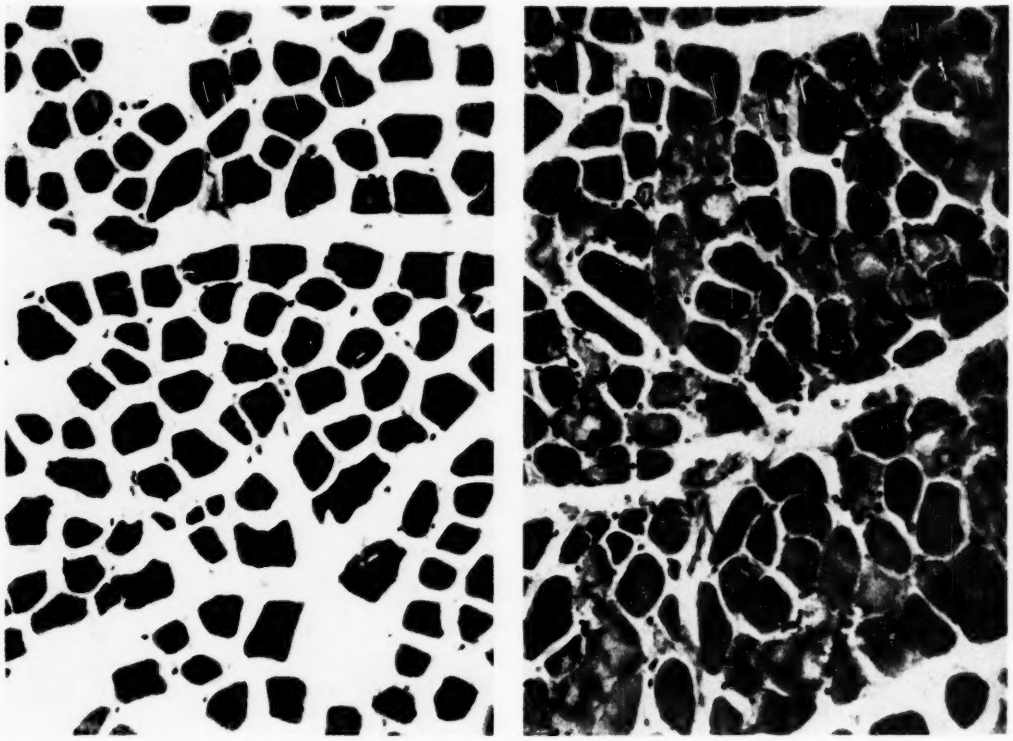


A B

FIG. 1. MUSCLE DAMAGE BY COLD

A. Section of muscle of right (unexposed) leg of rabbit whose left leg was immersed in water at 3°C. for 64 hours. Section taken at necropsy 30 days after exposure (×80).

B. Section of muscle of left (exposed) leg of same rabbit (×80).



A B

FIG. 2. MUSCLE DAMAGE BY COLD

A. Section of muscle of right (unexposed) leg of rabbit whose left leg was immersed in water at 3°C. for 45 hours. Section taken at necropsy 30 days after exposure (× 360).

B. Section of muscle of left (exposed) leg of same rabbit (× 360).

ability to spread toes was regained in a significantly shorter time by the animals receiving oxygen than by those without oxygen (Table II). No difference was detected between the exposed legs of rabbits breathing oxygen and air when exposures were for 24 or for 45 hours.

No difference in functional changes or recovery was observed between the control animals and those receiving cortisone.

Four rabbits confined in the exposure apparatus with legs dependent but not exposed to cold water developed edema and some diminution of ability to bear weight in both hind legs. The edema disappeared within 24 hours, more rapidly than in legs exposed to cold water. At no time was there any impairment of the ability to spread the toes. All measured functions returned to normal within four days after "exposure".

Room temperatures were kept at 23°C. or above during these experiments and no abnormal drop in body temperature was observed.

Pathological

Limbs of 56 rabbits were studied for pathological changes. Nineteen had received oxygen, 12 cortisone, and 25 were untreated. The time of exposure to cold varied from 20 to 64 hours. Thirty-five rabbits survived for 30 ± 1 days and were sacrificed. Of those exposed for 20 to 25 hours 15 of the 16 survived for 30 days. Of those exposed for 50 to 60 hours only 2 of 8 survived for 30 days. Generally speaking, only in animals dying within 48 hours after exposure was marked tissue edema noted grossly. Ulcers were an inconstant and uncommon finding and were believed, because of their location, to be on a traumatic

SUMMARY

1. Immersion foot was produced in 107 rabbits by exposing their left hind legs to water at 3°C. for times varying from 8 to 64 hours.

2. Functional changes resulted. These included inability to spread toes, inability to dorsiflex the foot, inability to hop normally, and decreased ability to bear weight on the left hind leg.

3. Pathological changes resulted in the muscle. These included basophilia, cellular infiltration, giant cell formation, fragmentation, abnormal variation of the size of muscle bundles, and edema.

4. The degree of functional and pathological change varied directly with the time of exposure.

5. There was an abrupt increase in both pathological and functional changes after 30 hours of exposure.

6. There was suggestive evidence that oxygen administration during exposure suppressed the functional changes, particularly of legs exposed to cold water for 30 hours. There was no evidence that oxygen breathing modified the pathological changes in muscle.

7. Cortisone appeared not to influence the functional or pathological changes in rabbit legs so exposed.

ACKNOWLEDGMENTS

We wish to thank Professor Carl Schmidt of the Department of Pharmacology for lending us laboratory space for these animal experiments, and D. W. T. Cochrane for valuable technical advice.

We wish to thank Professor F. D. W. Lukens, Director of the George S. Cox Medical Research Institute, University of Pennsylvania, who examined the kidneys.

We wish to thank Merck & Co. for the Cortone (cortisone) used in this study.

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THE DISTRIBUTION OF I^{131} LABELED HUMAN SERUM ALBUMIN INTRODUCED INTO ASCITIC FLUID: ANALYSIS OF THE KINETICS OF A THREE COMPARTMENT CATENARY TRANSFER SYSTEM IN MAN AND SPECULATIONS ON POSSIBLE SITES OF DEGRADATION

By SOLOMON A. BERSON and ROSALYN S. YALOW

(From the Radioisotope Unit of the Veterans Administration Hospital, Bronx, N. Y.)

(Submitted for publication July 20, 1953; accepted November 25, 1953)

The use of I^{131} labeled human serum albumin as a tracer has permitted the study of transfer of serum albumin between plasma and extravascular compartments. Distribution of intravenously administered iodoalbumin between plasma and ascitic fluid has been previously analyzed in terms of a simplified two compartment system (1, 2). In the present investigation iodoalbumin was administered intraperitoneally and the kinetics of distribution were analyzed by reference to a three compartment schematic model composed of ascitic fluid, plasma and extravascular spaces other than peritoneal cavity. The time course of excretion of the radioactive iodine released by degradation of the labeled albumin was also followed in an attempt to localize the site of metabolic breakdown of iodoalbumin.

METHODS

The subject of the distribution study was a 50-year-old white man, who, over the preceding eight months, had been subjected to repeated paracenteses for ascites secondary to Laennec's cirrhosis. Ten drops of Lugol's solution was administered three times a day throughout the period of observations to inhibit uptake by the thyroid of iodine released from the degraded iodoalbumin. Following administration of 112 μc of dialyzed I^{131}-tagged human serum albumin into the peritoneal cavity, ascitic fluid and heparinized plasma samples were obtained at almost daily intervals and complete urine collections were made. Under these conditions, loss of radioiodine from the body is almost entirely accounted for through renal excretion (2). Furthermore, owing to the relative rapidity of excretion as compared to degradation, the curve of urinary excretion of radioiodide may be considered as virtually identical with the curve of iodoalbumin degradation (2).

Radioactivity in plasma, ascitic fluid, and urine was assayed as previously described (2). Albumin concentrations in plasma and ascitic fluid were determined both by the micro-Kjeldahl method employing the technique of Kingsley (3) for the precipitation of proteins and by electrophoretic analysis. Previous observations (2), that at least 98 per cent of radioactivity in ascitic fluid and plasma is precipitable with cold 10 per cent trichloracetic acid, were confirmed. The patient did not have detectable proteinuria and radioactivity in the urine was not precipitated by trichloracetic acid even after carrier albumin was added.

The ascitic fluid volume was calculated from the 15-minute space of distribution of the I^{131} labeled albumin and was determined at the beginning and at the end of the experiment. Shaking and massage of the abdomen facilitated mixing. Plasma volume had been measured previously (2) with I^{131} labeled albumin (4) and was assumed to be unchanged.

SCHEMATIC MODEL

Following intraperitoneal injection, the fluid compartments are represented as components of a catenary system[1] in which the peritoneal cavity and extravascular space[2] (other than peritoneal cavity) are end compartments communicating with the intervening plasma compartment, as shown in Figure 1. Although it is presumed that albumin leaves the blood stream by passage through the capillary walls, it is probable that return is chiefly by way of the lymphatics under normal conditions. The boundary for return from ascitic fluid to plasma would then involve an intervening shunt, the lymphatic vessels, which is considered a part of the extravascular space. In addition, another shunt space is the right pleural cavity which may receive albumin from the peritoneal cavity in a one-way transfer. However, the quantity of albumin within these shunts at any time is small compared to that in any of the three major compartments and is therefore neglected.

Following intravenous administration of I^{131} albumin to subjects without abnormal fluid accumulations, such as ascites, a 2 component curve of disappearance from plasma was previously observed in most instances (2). This curve possibly represents a mammillary system in which the central reservoir, plasma, is in communication with

[1] The term catenary is used by Sheppard & Householder (5) to describe a series of compartments communicating endwise with each other. The term mammillary is used to describe a number of peripheral compartments in communication with a central reservoir.

[2] The extravascular space of serum albumin distribution is thought to be composed chiefly of fluid in the interstitial (extracellular) space and lymphatic channels but the possibility of some intracellular localizations is not excluded.

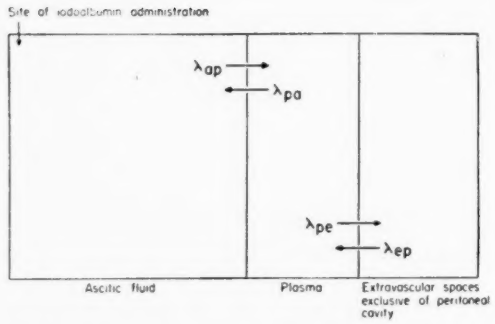

FIG. 1. SCHEMATIC MODEL FOR TRANSFER OF I^{131} ALBUMIN ADMINISTERED INTRAPERITONEALLY

two groups of extravascular spaces with different average net transfer rates. In the present study it was felt that consideration of more than a single rate of transfer between plasma and extravascular space would introduce unresolvable complications into the analysis. Therefore, the extravascular spaces were assumed to comprise a single compartment communicating with plasma by a single transfer rate representing an average of all such transfer rates.

Let A, P, and E be the quantities of exchangeable endogenous albumin in ascitic fluid, plasma and extravascular space, respectively, A^*_0 be the total amount of I^{131} labeled albumin introduced into the ascitic fluid compartment and A^*, P^*, and E^* be the fractions of A^*_0 in the respective compartments at any time, t. Let λ_{ap}, λ_{pa}, λ_{pe}, and λ_{ep} be the rate constants of transfer between the compartments in the directions indicated by the order of the subscript designations, and A^*_∞, P^*_∞, and E^*_∞ be the fractions of A^*_0 in the respective compartments at distribution equilibrium.[3]

In the "steady state," the quantities of endogenous albumin in the various compartments remain unchanged. Assuming that the amounts being transferred in each direction remain constant and that the labeled and unlabeled molecules behave identically, then

$$\frac{dA^*}{dt} = -\lambda_{ap}A^* + \lambda_{pa}P^* \qquad (1)$$

$$\frac{dP^*}{dt} = -\lambda_{pa}P^* + \lambda_{ap}A^* - \lambda_{pe}P^* + \lambda_{ep}E^* \qquad (2)$$

$$\frac{dE^*}{dt} = -\lambda_{ep}E^* + \lambda_{pe}P^* \qquad (3)$$

Employing the conditions that

$$A^* = A^*_0,\ P^* = 0,\ E^* = 0 \qquad \text{when } t = 0$$

and

$$A^* = A^*_\infty,\ P^* = P^*_\infty,\ E^* = E^*_\infty \qquad \text{when } t = \infty,$$

the system of simultaneous linear differential equations, (1) (2) (3), is solved for A^*, P^* and E^* by standard methods (Appendix A).

$$A^* = A^*_\infty + c_1e^{-\lambda_1 t} + c_2e^{-\lambda_2 t} \qquad (4)$$

$$P^* = P^*_\infty - (k_1 + c_1)e^{-\lambda_1 t} - (k_2 + c_2)e^{-\lambda_2 t} \qquad (5)$$

$$E^* = E^*_\infty + k_1e^{-\lambda_1 t} + k_2e^{-\lambda_2 t} \qquad (6)$$

where

$$\lambda_1,\lambda_2 = \tfrac{1}{2}\{(\lambda_{pa} + \lambda_{pe} + \lambda_{ap} + \lambda_{ep}) \pm [(\lambda_{pa} + \lambda_{pe} + \lambda_{ap} + \lambda_{ep})^2 - 4(\lambda_{ap}\lambda_{pe} + \lambda_{ep}\lambda_{pa} + \lambda_{ap}\lambda_{ep})]^{\frac{1}{2}}\}$$

$$c_1 = \frac{\lambda_2(A^*_0 - A^*_\infty) - \lambda_{ap}A^*_0}{\lambda_2 - \lambda_1} \qquad k_1 = -\frac{\lambda_2E^*_\infty}{\lambda_2 - \lambda_1}$$

$$c_2 = -\frac{\lambda_1(A^*_0 - A^*_\infty) - \lambda_{ap}A^*_0}{\lambda_2 - \lambda_1} \qquad k_2 = \frac{\lambda_1E^*_\infty}{\lambda_2 - \lambda_1}$$

ANALYSIS OF OBSERVED DATA

The amounts of I^{131}-albumin in the ascitic fluid and plasma compartments at any time, t, were calculated as the products of the compartment volumes and the respective concentrations of I^{131}-albumin. The previously determined plasma volume was assumed to remain unchanged throughout the period of observations. However, over this 22-day period, the ascitic fluid volume increased from 14.5 to 21 liters. This increase, averaging 295 milliliters per day, or between 1½ per cent and 2 per cent per day, was assumed to proceed linearly with time, since the subject's weight increased gradually over this period. This necessary approximation probably did not introduce an error of more than 5 per

TABLE I

*Ascitic fluid and plasma concentrations of serum albumin**

Day of study	Ascitic fluid *Gm./100 ml.*	Plasma *Gm./100 ml.*
5	1.50	2.95
6	1.50	2.85
7	1.51	
8	1.58	
12	1.49	3.04
13	1.44	3.00
14	1.48	2.98
15		2.98
16	1.45	
19	1.51	2.85
20	1.47	3.05

* These values were obtained from Kjeldahl determinations. Electrophoresis gave absolute values which were somewhat lower but with the same ratio between plasma and ascitic fluid.

[3] In this paper distribution equilibrium is defined as a dynamic state in which transfer of the labeled albumin from one compartment to another per unit time is balanced by transfer of an equal amount in the reverse direction.

cent into the calculation of total ascitic fluid I^{131}-albumin at any time.

Then,

P^* = plasma volume (ml.) × fraction of A^*_0/ml. plasma

A^* = ascitic fluid volume (ml.) × fraction of A^*_0/ml. ascitic fluid

ascitic fluid volume (ml.) = 14,500 + 295t where t is the number of days from the beginning of the study.

The total quantity of extravascular iodoalbumin was calculated as the total iodoalbumin remaining within the body, less the sum of ascitic fluid and plasma iodoalbumin. The amount retained within the body ($A^*_{0\ ret}$) at any time, t, was taken as the dose administered less the cumulative renal excretion up to that time.

Then,

$$A^*_{0\ ret} = A^*_0 - U^*$$
$$E^* = A^*_{0\ ret} - (P^* + A^*)$$

where U^* is the cumulative urine excretion.

Direct determinations of extravascular I^{131}-albumin concentration were not feasible. However, such determinations would not have permitted estimates of total extravascular I^{131}-albumin since there are wide variations in albumin concentration in different portions of this compartment and the volume of each portion is not known. The concentrations of albumin in ascitic fluid and plasma remained essentially constant (Table I). When the relative specific activities of the ascitic fluid, plasma and extravascular space were plotted as a function of time on semi-

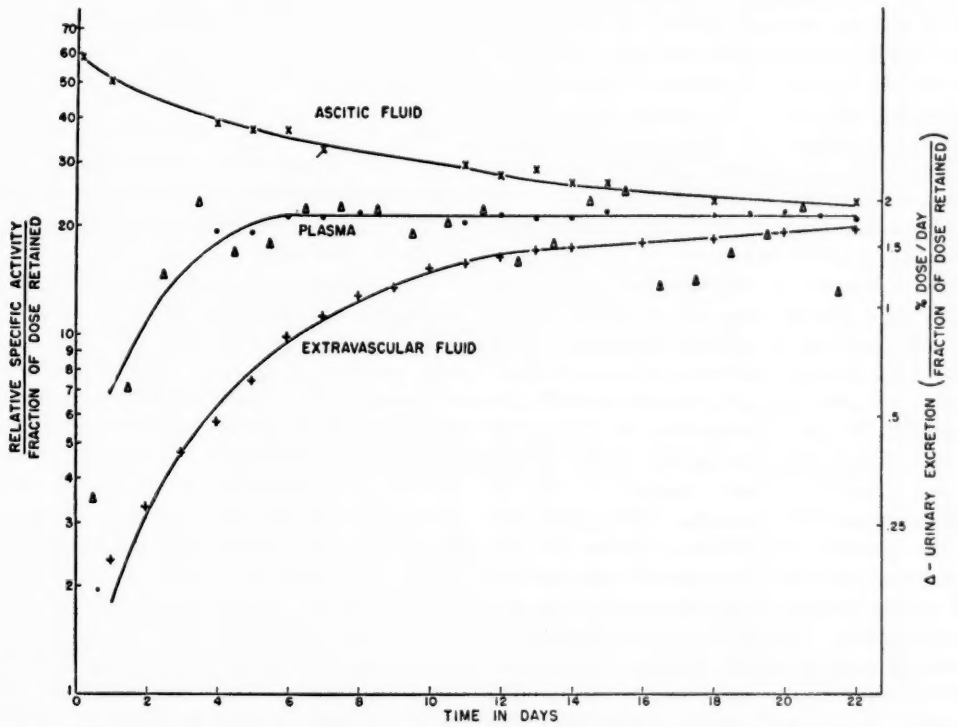
ASCITIC FLUID
PLASMA
EXTRAVASCULAR FLUID
RELATIVE SPECIFIC ACTIVITY / FRACTION OF DOSE RETAINED
Δ - URINARY EXCRETION (% DOSE / DAY / FRACTION OF DOSE RETAINED)
TIME IN DAYS

FIG. 2. RELATIVE SPECIFIC ACTIVITIES OF I^{131} ALBUMIN IN ASCITIC FLUID, PLASMA AND EXTRAVASCULAR FLUID AND URINARY EXCRETION OF RADIOACTIVITY AS A FUNCTION OF TIME FOLLOWING INTRAPERITONEAL INJECTION OF I^{131} ALBUMIN

Points on the ascitic fluid (A) and plasma (P) curves are derived from experimentally determined values. Points on the extravascular fluid curve were calculated as described in Figure 3 and the text. Total endogenous albumin in plasma was 137 grams, in extravascular fluid 157 grams, and in ascitic fluid 217 grams at onset increasing to 316 grams at conclusion of study.

plasma and extravascular space, the relative constancy of λ_{ps}, λ_{pe}, and λ_{ep} may be accepted. But λ_{ap} must have decreased proportionately as the ascitic fluid albumin increased. The constancy of λ_1 and λ_2, as implied in Figure 3, is, therefore, only approximate. This lack of constancy is difficult to detect, however, since a 45 per cent decrease in λ_{ap} produces only a 14 per cent change in λ_1 and less than a 1 per cent change in λ_2.

The steady state condition that $\lambda_{ps}P = \lambda_{ap}A$ is also not satisfied when there is a net increase in ascitic fluid albumin. Since this increase averaged about 4.4 grams daily, the experimental condition for the albumin transferred per day is $\lambda_{ps}P = \lambda_{ap}A + 4.4$. The value obtained for the rate of albumin transfer from plasma to ascitic fluid is then approximately 24 grams per day (Appendix B) and that for transfer in the reverse direction about 20 grams per day.

From these considerations it may be concluded that, because of the approximations required by the absence of steady state conditions (which rarely exist in disease states), the values derived even from a reasonably complete analysis of available data, can be considered only roughly quantitative. Any further simplification of the reference scheme is likely to yield even less precise quantitative results. An example of this is presented in the values obtained for albumin transfer between plasma and extravascular fluid. It is calculated that approximately 30 grams daily passed in each direction between plasma and extravascular fluid (Appendix B). However, following intravenous administration of iodoalbumin in subjects without ascites, the initial fall in plasma concentration is of the order of 5 per cent per hour (2, 4), which indicates a transfer into extravascular fluid of over 100 grams albumin per day. It is possible that exchange between plasma and extravascular space was abnormally low in this cirrhotic subject because of increased tissue tension resulting from edema. Yet large errors can result from the simplifying assumption of a single extravascular compartment communicating with plasma at a single transfer rate. For example, a small extravascular compartment equilibrating rapidly with plasma could well escape detection in this type of analysis and yet be responsible for a quantitatively greater albumin traffic to and from plasma than all the rest of extravascular space.

From the theoretical point of view a complete

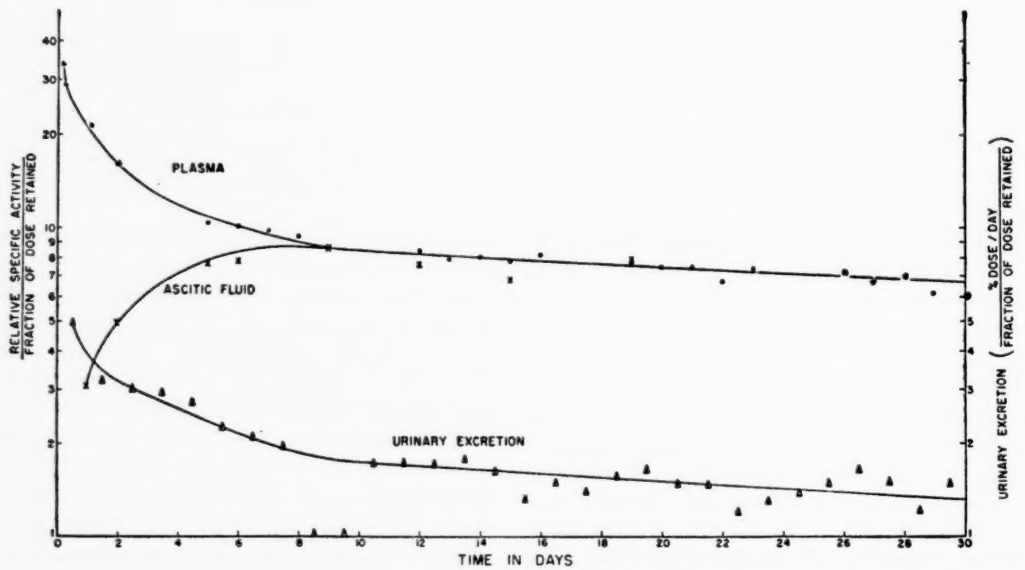

FIG. 4. SPECIFIC ACTIVITIES OF I^{131} ALBUMIN IN ASCITIC FLUID AND PLASMA AND URINARY EXCRETION OF RADIOACTIVITY AS A FUNCTION OF TIME FOLLOWING THE INTRAVENOUS ADMINISTRATION OF I^{131} ALBUMIN

kinetic analysis of the closed steady state schematic model requires only the curve of radioactivity concentration in the ascitic fluid and a knowledge of the total amounts of albumin in the ascitic fluid and plasma. In practice, however, even small experimental errors are sufficient to lead to gross miscalculations when such limited data alone are available. The failure to obtain immediate complete mixing in the ascitic fluid may lead to an early steep fall in concentration of radioactivity which, if observations were confined to this compartment alone, would be interpreted as indicating a greater transfer rate than actually exists. On the other hand, as noted above, a slight experimental error may also obscure a rapid transfer component into a small compartment. Extending observations to the receiving compartments aids in discriminating between experimental errors and variations arising from the specific kinetic characteristics of the system.

SITE OF IODOALBUMIN DEGRADATION

It has previously been noted that the curve of renal excretion of radioactivity is virtually identical with the curve of degradation of iodoalbumin (2). If one compartment were the sole or major site of degradation, it would be expected that the renal excretion of radioactivity would be more or less directly proportional to the radioactivity present in the degrading compartment. In Figure 4 are shown the results of a previous experiment in the same subject where iodoalbumin was administered intravenously. Since ascitic fluid volumes were not measured directly, calculated values for the extravascular space are not shown. The renal excretion follows more closely the specific activity of the plasma than that of the ascitic fluid. In three other cirrhotic subjects with ascites where ascitic fluid concentrations of radioactivity were not assayed, but may be presumed to have shown generally similar increasing curves, the plasma and urine curves were similar to those shown here. These patterns indicate that degradation is not proportional to ascitic fluid radioactivity and, therefore, that the ascitic fluid is not a major site of degradation. A similar conclusion can be inferred from the present study, since renal excretion increased as ascitic fluid radioactivity decreased. There is also an apparently closer correlation between the radioactivity in the plasma and renal excretion than between the radioactivity in extravascular space and renal excretion. This suggests that extravascular space as a whole is also not a major site of degradation. However, the conclusion that the parallelism of the plasma and urine curves indicates the plasma as the site of iodoalbumin catabolism must be avoided. An alternative interpretation is that a certain segment of the extravascular space in fairly rapid distribution equilibrium with the plasma may be the site of degradation. Previous studies (2) have shown that there is some extravascular compartment which exchanges rapidly with plasma. Owing to some lag in excretion of degraded radioactivity and the presence of small amounts of protein-free radioactivity even after prolonged dialysis, the renal excretions over the first few hours cannot be taken so seriously as to discriminate between the plasma and the rapidly equilibrating extravascular compartments. The possibility that this extravascular compartment is located in an intracellular site cannot be excluded.

DISCUSSION

A general mathematical approach to the problem of tracer mixing in multicompartment systems has been presented by Sheppard and Householder (5). The present study illustrates the practical difficulties encountered in attempting to apply a theoretical analysis to a specific problem, that of the distribution of intraperitoneally administered I^{131} tagged albumin. The failure of the biological system to remain conveniently constant introduced discrepancies of moderate degree into the derived data. The simplifying reduction of all extravascular spaces into a single compartment necessitated by the lack of information regarding the various extravascular compartments and the infeasibility of repeated sampling of them may be responsible for errors of greater magnitude. It would appear to be even less justified to neglect the extravascular compartments entirely as well as the consideration of changing volumes, as in the approach of Schoenberger, Kroll, Sakamoto, and Kark (1). These authors have concluded that alterations in permeability are responsible for

changes in albumin transfer rates observed in cirrhotic patients under therapy (6). In view of the obstacles which oppose the accurate evaluation of these transfer rates it seems a bit hazardous to rely too heavily on the quantitative aspects of data calculated by reference to oversimplified systems.

In spite of this conservative approach to the significance of values obtained for albumin exchange among the various compartments, certain quantitative considerations are in order. In normal subjects, extravascular space contains approximately 150 to 200 grams of albumin (2). Following intravenous administration of I^{131} albumin to non-edematous subjects, the half time for equilibration with the major portion of this extravascular albumin is about 24 hours (2). It thus appears that the rate of transfer of plasma albumin to most of the extravascular compartment is normally only of the order of five times the rate of transfer of plasma albumin to ascitic fluid (24 grams per day) observed in the cirrhotic subject of the present study. Krogh (7) has estimated the total surface area of capillaries throughout the body to be about 6000 square meters. Since the total surface area of the peritoneal capillaries probably does not approach that value within a factor of more than one hundred, a conservative estimate must grant an albumin transfer rate per unit capillary surface area at least 20 times greater through the average peritoneal capillary of this subject than through the normal average extraperitoneal capillary. Each square centimeter of the peritoneal membrane itself, which cannot have a total surface area of much more than 1 square meter, is therefore the site of even considerably greater transfer activity. Data for the normal subject would appear difficult to obtain without altering normal physiological conditions.

The continued accumulation of ascitic fluid albumin in cirrhotic subjects indicates either an abnormal increase in the rate of transfer from plasma to ascitic fluid or a decrease in the rate of removal from the peritoneal cavity or both. We are not aware of any evidence bearing on obstruction of the lymphatics which drain the peritoneal cavity in cirrhosis, while the high portal venous pressure present in this condition suggests a mechanism for the first alternative. Then, fluctuations in the rate of ascitic fluid accumulation in cirrhosis may depend chiefly on changes in portal capillary pressure (as is suggested by the occasional success of shunting operations) rather than on any change in permeability of the capillaries. It would be of interest to investigate the rate of albumin transfer in nephrotic ascites where portal venous pressure is not elevated.

It has been reported that, after intraperitoneal administration of iodoalbumin, the specific activity of ascitic fluid remains higher than that of plasma (8). This relationship is more apparent than true and in the present case owes itself to a half time of over five days for the slow component of the distribution curves. A month or more may be required for essentially complete distribution of iodoalbumin administered intraperitoneally to subjects with marked ascites.

The problem of the site of degradation remains unsolved from the data at hand. It appears as if the iodoalbumin is subject to degradation in the plasma itself or in a rapidly equilibrating extravascular compartment. Attempts to study the proteolytic effect of plasma on iodoalbumin *in vitro* have thus far given equivocal results.

It has previously been emphasized (2) that the metabolic degradation of I^{131} labeled albumin has not as yet been conclusively demonstrated to be identical with that of endogenous serum albumin. Conclusions regarding iodoalbumin metabolism can therefore only tentatively be suggested as reflecting the fate of endogenous albumin.

CONCLUSIONS

1. Following intraperitoneal administration of I^{131} labeled human serum albumin, the kinetics of distribution have been analyzed mathematically with reference to a three compartment schematic model composed of ascitic fluid, plasma, and extravascular fluid.

Errors of the analysis arising from non-steady state conditions and deficiencies in the schematic model are discussed.

2. It was demonstrated that essentially complete distribution of intraperitoneally administered I^{131} labeled albumin may require almost a month in the presence of marked ascites. The rate of albumin transfer from plasma to ascitic fluid was calculated to be approximately 24

grams per day. During a 22-day period of observations the ascitic fluid showed a net increase in albumin content which averaged 4.4 grams per day. Evidence is presented which indicates that this increase represented newly formed albumin rather than a depletion of exchangeable albumin stores in plasma and extravascular fluid.

3. From considerations of relative available capillary surface areas it is concluded that the peritoneal capillary of the cirrhotic subject of the present study permitted a much greater albumin transfer out of plasma than the average capillary elsewhere in the body.

4. From a comparison of the time course of renal excretion of radioactivity with the curves of I^{131} labeled albumin in ascitic fluid, plasma and extravascular fluid following intraperitoneal and intravenous administration of the labeled albumin, the site of degradation of iodoalbumin has been inferred to be the circulating plasma or some extravascular compartment equilibrating rapidly with plasma.

APPENDIX A[4]

Solution of the differential equations *1*, *2*, *3*

$$\frac{dA}{dt} = -\lambda_{ap}A + \lambda_{pa}P \qquad (1)$$

$$\frac{dP}{dt} = -\lambda_{pa}P + \lambda_{ap}A - \lambda_{pe}P + \lambda_{ep}E \qquad (2)$$

$$\frac{dE}{dt} = -\lambda_{ep}E + \lambda_{pe}P \qquad (3)$$

Differentiating (*1*) and substituting the value for $\frac{dP}{dt}$ given in (*2*) gives

$$\frac{d^2A}{dt^2} = -\lambda_{ap}\frac{dA}{dt} - \lambda_{pa}P\,(\lambda_{pa} + \lambda_{pe}) + \lambda_{ap}\lambda_{pa}A + \lambda_{pa}\lambda_{ep}E \qquad (4)$$

Substituting the value for $\lambda_{pa}P$ determined from (*1*), differentiating and collecting terms,

$$\frac{d^3A}{dt^3} + \frac{d^2A}{dt^2}(\lambda_{ap} + \lambda_{pa} + \lambda_{pe}) + \frac{dA}{dt}(\lambda_{ap}\lambda_{pe}) = \lambda_{pa}\lambda_{ep}\frac{dE}{dt} \qquad (5)$$

[4] To avoid confusion the asterisks have been deleted although only the labeled species is under consideration here.

Substituting the value for $\frac{dE}{dt}$ determined from the relation, $\frac{dP}{dt} + \frac{dA}{dt} + \frac{dE}{dt} = 0$, and the value for $\frac{dP}{dt}$ determined from the derivative of (*1*) and collecting terms,

$$\frac{d^3A}{dt^3} + \frac{d^2A}{dt^2}(\lambda_{ap} + \lambda_{pa} + \lambda_{ep} + \lambda_{pe}) + \frac{dA}{dt}(\lambda_{pa}\lambda_{ep} + \lambda_{ap}\lambda_{ep} + \lambda_{ap}\lambda_{pe}) = 0 \qquad (6)$$

To solve for A we find the value of λ so that (*6*) will be satisfied by a solution of the form

$$A = ce^{\lambda t} \qquad (7)$$

Differentiating (*7*) and substituting in (*6*) reduces (*6*) to a cubic equation in λ which has three roots,

$$\lambda_1,\lambda_2 = \tfrac{1}{2}\{-(\lambda_{ap} + \lambda_{pa} + \lambda_{ep} + \lambda_{pe}) \pm [(\lambda_{ap} + \lambda_{pa} + \lambda_{ep} + \lambda_{pe})^2 - 4(\lambda_{pa}\lambda_{ep} + \lambda_{ap}\lambda_{ep} + \lambda_{ap}\lambda_{pe})]^{\frac{1}{2}}\} \qquad (8)$$

$$\lambda_3 = 0$$

The general solution for A is, then,

$$A = c_1e^{-\lambda_1 t} + c_2e^{-\lambda_2 t} + c_3 \qquad (9)$$

which contains three essential arbitrary constants and in which the values for λ are the negatives of those given above. Similarly

$$P = m_1e^{-\lambda_1 t} + m_2e^{-\lambda_2 t} + m_3 \qquad (10)$$

$$E = k_1e^{-\lambda_1 t} + k_2e^{-\lambda_2 t} + k_3 \qquad (11)$$

At t_∞, A, P, and E take on their ∞ values, Therefore $c_3 = A_\infty$, $m_3 = P_\infty$, $k_3 = E_\infty$. Since $A_0 = A + P + E = A_\infty + P_\infty + E_\infty$, the sum of equations (*9*), (*10*), (*11*) gives

$$(c_1 + m_1 + k_1)\,e^{-\lambda_1 t} + (c_2 + m_2 + k_2)\,e^{-\lambda_2 t} = 0, \qquad (12)$$

which is satisfied for all values of t only by

$$c_1 + m_1 + k_1 = 0$$
$$c_2 + m_2 + k_2 = 0$$

Equations (*9*), (*10*), (*11*) may then be rewritten in the form given in the body of the paper,

$$A = A_\infty + c_1e^{-\lambda_1 t} + c_2e^{-\lambda_2 t} \qquad (13)$$

$$P = P_\infty - (k_1 + c_1)e^{-\lambda_1 t} - (k_2 + c_2)e^{-\lambda_2 t} \qquad (14)$$

$$E = E_\infty + k_1e^{-\lambda_1 t} + k_2e^{-\lambda_2 t} \qquad (15)$$

From (*1*) and (*13*)

$$\frac{dA}{dt} = -\lambda_{ap}A + \lambda_{pa}P = -\lambda_1 c_1 e^{-\lambda_1 t} - \lambda_2 c_2 e^{-\lambda_2 t} \quad (16)$$

at t = 0

$$\frac{dA}{dt} = -\lambda_{ap}A_0 = -\lambda_1 c_1 - \lambda_2 c_2 \quad (17)$$

Similarly from (*2*) and (*14*) at t = 0

$$\frac{dP}{dt} = \lambda_{ap}A_0 = \lambda_1(k_1 + c_1) + \lambda_2(k_2 + c_2) \quad (18)$$

Then from (*17*) and (*18*),

$$k_1 = -\frac{\lambda_2}{\lambda_1} k_2 \quad (19)$$

From (*15*), at t = 0

$$E_0 = 0 = E_\infty + k_1 + k_2 \quad (20)$$

Solving for k_1 and k_2 from (*19*) and (*20*)

$$k_1 = -\frac{\lambda_2 E_\infty}{\lambda_2 - \lambda_1} \quad (21)$$

$$k_2 = \frac{\lambda_1 E_\infty}{\lambda_2 - \lambda_1} \quad (22)$$

From (*13*), at t = 0

$$A_0 = A_\infty + c_1 + c_2 \quad (23)$$

From (*17*) and (*23*),

$$c_1 = \frac{\lambda_2(A_0 - A_\infty) - \lambda_{ap}A_0}{\lambda_2 - \lambda_1} \quad (24)$$

$$c_2 = -\frac{\lambda_1(A_0 - A_\infty) - \lambda_{ap}A_0}{\lambda_2 - \lambda_1} \quad (25)$$

APPENDIX B[5]

Evaluation of the rate constants of iodoalbumin transfer from the values obtained in the graphical analysis

It is desired to express each rate constant in terms of the coefficients and exponents of equations (*13*), (*14*), and (*15*) in Appendix A.

Substituting in (*1*), the values for A, P, and $\frac{dA}{dt}$ derived from (*13*) and (*14*) of Appendix A,

$$-c_1\lambda_1 e^{-\lambda_1 t} - c_2\lambda_2 e^{-\lambda_2 t} = -\lambda_{ap}(A^*_\infty + c_1 e^{-\lambda_1 t} + c_2 e^{-\lambda_2 t}) + \lambda_{pa}\{P^*_\infty - (c_1 + k_1)e^{-\lambda_1 t} - (k_2 + c_2)e^{-\lambda_2 t}\} \quad (1)$$

Collecting terms,

$$e^{-\lambda_1 t}\{-c_1\lambda_1 + c_1\lambda_{ap} + (c_1 + k_1)\lambda_{pa}\} = e^{-\lambda_2 t}\{c_2\lambda_2 - c_2\lambda_{ap} - (k_2 + c_2)\lambda_{pa}\} - \lambda_{ap}A^*_\infty + \lambda_{pa}P^*_\infty \quad (2)$$

Since, from the equilibrium conditions,

$$-\lambda_{ap}A^*_\infty + \lambda_{pa}P^*_\infty = 0,$$

equation (*2*) is satisfied for all values of t only if

$$c_1\lambda_{ap} + (c_1 + k_1)\lambda_{pa} - c_1\lambda_1 = 0 \quad (3)$$

$$c_2\lambda_{ap} + (c_2 + k_2)\lambda_{pa} - c_2\lambda_2 = 0 \quad (4)$$

Solving for λ_{pa} and λ_{ap},

$$\lambda_{pa} = \frac{c_1 c_2(\lambda_1 - \lambda_2)}{c_2 k_1 - c_1 k_2} \quad (5)$$

$$\lambda_{ap} = \lambda_1 - \frac{(c_1 + k_1)c_2(\lambda_1 - \lambda_2)}{c_2 k_1 - c_1 k_2}$$

Similarly,

$$\lambda_{pe} = \frac{k_1 k_2(\lambda_1 - \lambda_2)}{c_1 k_2 - c_2 k_1} \quad (7)$$

$$\lambda_{ep} = \lambda_1 - \frac{(k_1 + c_1)k_2(\lambda_1 - \lambda_2)}{c_1 k_2 - c_2 k_1} \quad (8)$$

The values obtained by graphical analysis (Figure 3),

$\lambda_1 = .133$ day^{-1}; $\lambda_2 = .562$ day^{-1};
$c_1 = .465$; $c_2 = .060$; $k_1 = -.390$, $k_2 = .095$

are substituted in (*5*), (*6*), (*7*), and (*8*). Then

$$\lambda_{ap} = .102 \text{ day}^{-1}$$
$$\lambda_{pa} = .178 \text{ day}^{-1}$$
$$\lambda_{pe} = .236 \text{ day}^{-1}$$
$$\lambda_{ep} = .178 \text{ day}^{-1}$$

When these values for the transfer rate constants are substituted in equation (*8*) of Appendix A, the values obtained for λ_1 and λ_2,

$$\lambda_1 = .130 \text{ day}^{-1}$$
$$\lambda_2 = .564 \text{ day}^{-1}$$

agree with the graphical values used.

Employing the values for A, P, and E at the beginning of the experiment, albumin transfer from ascitic fluid to plasma is, then,

$\lambda_{ap}A = (.102 \text{ day}^{-1})(218 \text{ Gm.}) = 22 \text{ Gm./day}$

Similarly,

Albumin transfer from plasma to ascitic fluid = (.178)(137) = 24 Gm./day

[5] The asterisks have been reintroduced here to differentiate labeled from unlabeled albumin.

Albumin transfer E.V. space to plasma = (.178)(157) = 28 Gm./day

Albumin transfer plasma to E.V. space = (.236)(137) = 32 Gm./day

Discrepancies arising from the absence of strict steady state conditions are discussed in the body of the paper.

ACKNOWLEDGMENTS

We are indebted to Dr. Lynn Fulkerson for obtaining the ascitic fluid samples and to Mr. Paul Newman for the illustrations. Thanks are also due to Mrs. Florence Cavanagh and Mrs. Frieda Steiner for secretarial assistance.

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THE CONCENTRATION AND SIGNIFICANCE OF THE BUTANOL-EXTRACTABLE I^{131} OF SERUM IN PATIENTS WITH DIVERSE STATES OF THYROIDAL FUNCTION

By SIDNEY H. INGBAR,[1] NORBERT FREINKEL,[1] PAUL D. HOEPRICH, AND JOHN W. ATHENS

(From the Department of Biophysics, Army Medical Service Graduate School, Walter Reed Army Medical Center, Washington 12, D. C.)

(Submitted for publication September 28, 1953; accepted November 12, 1953)

Thyroxine, if not truly the active thyroid hormone (1, 2) is clearly the principal iodine-containing compound released by the thyroid gland, and ordinarily comprises the largest fraction of the plasma's protein-bound iodine (3–6). Many investigators have, therefore, attempted to assess thyroidal function by measuring the concentration of protein-bound I^{131} (PBI^{131}) in the plasma following the administration of radioactive iodine (7–11). The PBI^{131} (12), or even the total radioactivity in the plasma (13, 14), determined several days after the administration of the tracer, reflects the radioactivity of circulating thyroxine, except in patients who have received large doses of I^{131} (15).

It has been shown that the hormonal iodine which is extractable from serum with butanol and not re-extractable with alkali (hereafter referred to as the butanol-extractable iodine) is composed largely of thyroxine. Its concentration in serum differs significantly from that of the protein-bound iodine (16).

The present communication reports the results in 109 patients with varying states of thyroidal function in whom the plasma's concentration of radioactive butanol-extractable iodine was determined both one and three days after the administration of I^{131}. From a correlation of these results with measurements of the amount of I^{131} accumulated by the thyroid gland, an estimate of both the amount and the rate of turnover of glandular hormone has been made.

MATERIALS AND METHODS

The present study includes all patients referred to the Department of Biophysics, Army Medical Service Graduate School, Walter Reed Army Medical Center during the period between January, 1952 and June, 1952 for the determination of 24-hour thyroidal uptake of radioactive iodine. Since no attempt was made to select cases within this group, which included dependents as well as military personnel, the population studied is probably representative of that referred to the thyroid clinic of any large hospital. Each patient was questioned and examined by one or another of the authors, and a diagnostic impression was recorded prior to the administration of I^{131}. This record, together with the result of the iodine-uptake test, was reviewed by one of the authors (S. H. I.), and each patient was assigned a definite diagnosis before the results of the butanol-extraction procedure were known. Prolonged observation of the patients, in an attempt to ascertain the accuracy of the diagnosis, was usually impossible.

A standard amount (50 to 100 μc) of I^{131}, free of carrier, was administered orally to each patient. The patient returned 24 hours later for the determination of the thyroidal uptake of the tracer. This was carried out with a shielded Geiger-Mueller tube (bismuth-sputtered-cathode) placed 20 cm. from the neck. The radioactivity in the neck was compared with that of a 50 ml. volumetric flask, similarly positioned, containing an amount of I^{131} equal to the dose administered. In most patients the thyroidal content of I^{131} was also determined 48 and 72 hours after the administration of the tracer. The following convention was adopted as an index of the preponderance of either accumulation of radioiodine or the release of radioactive hormone during the interval between 24 and 72 hours after the administration of I^{131}. When the thyroidal uptake displayed a progressive increase during this period, the 24-hour uptake was subtracted from the 72-hour uptake and the difference ("net change") was given a positive sign. When the uptake decreased at any time during the period, the lowest value was subtracted from the highest and the difference was given a negative sign.

During the 24 and 72-hour visits, blood was drawn for the measurement of the concentration of butanol-extractable I^{131} (BEI^{131}). Separation of the serum was carried out as soon as clot retraction had occurred. In virtually all cases, the analysis was begun on the same day the blood was drawn.

The method used in the extraction of thyroxine from serum was an adaptation of that described by Man, Kydd, and Peters (16). Five cc. of serum were brought to a pH of approximately 3.5 with 10 per cent H_2SO_4, using

[1] Present address: Thorndike Memorial Laboratory, Boston City Hospital, Boston 18, Mass.

a few drops of bromphenol-blue as an internal indicator. Twenty-five cc. of n-butyl alcohol were then added in a fine stream, while the serum was continuously agitated. The resulting mixture was centrifuged at 2500 r.p.m for 8 minutes. The supernatant butanol was transferred to a 125 ml. centrifuge separatory funnel. The precipitated protein was successively re-extracted with 20 cc. and 15 cc. portions of butanol. The three butanol extracts were combined within the separatory funnel, vigorously extracted three times with 60 cc. of Blau's solution (4 N NaOH containing 5 per cent Na_2CO_3). Slow centrifugation (300 r.p.m.) for 5 minutes was used to break the emulsion which invariably formed. The alkalinized, washed butanol extract was transferred to a 150 cc. distilling flask and was distilled to dryness *in vacuo* at a temperature not exceeding 40°C. The dried residue was dissolved in 1.5 to 2.0 cc. of butanol or water and transferred to weighed planchets. Dilute HCl was added in order to reduce the alkalinity, and thereby to diminish the hygroscopic properties of the mixture. When the residue was taken up in water, an immiscible layer appeared, which contained almost all the bromphenol-blue and most of the thyroxine. Radioactivity in this phase was 3 to 8 times as great as in the aqueous phase. The chemical nature of this immiscible layer has not been determined. It does not originate from serum, since it can be demonstrated when saline, with or without added thyroxine, is extracted, and presumably, therefore, arises from either the apparatus or the reagents.

In the determination of the concentration of BEI^{131} in erythrocytes, blood was drawn into a tube containing dried heparin. The erythrocytes were separated from the plasma and washed three times with normal saline solution. Five cc. of packed cells were laked with distilled water. Concomitant analyses for the concentration of BEI^{131} in plasma and laked erythrocytes were then carried out as above.

The radioactivity of the samples was measured with an end-windowed (mica 1.4 to 1.8 mg. per cm.^{2}) Geiger-Mueller tube and an automatic sample changer. At least 24,000 total counts were obtained for each serum sample. A correction, based on mass, was made in the observed radioactivity of each sample. Since, in the average euthyroid patient, the total thyroxine radioactivity in each sample of serum was approximately two-thirds of background radiation, approximately 9500 disintegrations were observed. The probable error of the determined radioactivity was therefore approximately 1 per cent. The accuracy of the counting procedure was considerably greater in hyperthyroid patients, in whom the concentration of radioactive thyroxine was usually markedly increased.

RESULTS

In an attempt to evaluate the analytic procedure described above, the recovery from serum of radioactive potassium iodide, diiodotyrosin,[2] and thyroxine[2] was determined (Table I). From 88 to 97 per cent of radioiodine added as thyroxine was recovered from the final residue, whereas I^{131} added as either potassium iodide or diiodotyrosine was virtually excluded.

[2] Obtained from Abbott Laboratories, Chicago, Illinois.

TABLE I

Recovery of radioactive thyroxine, diiodotyrosine, and potassium iodide during butanol-extraction procedure

Radioactive substance added	Number of experiments	Per cent of added radioactivity in butanol extract*		
		Prior to alkaline wash	Following alkaline wash	Following distillation
		%	%	%
Thyroxine	8	99.4	97.2	94.3
Diiodotyrosine	4	47.1	0.05	0.02
Potassium iodide	6	80.5	0.03	0.01

* Average values.

In both butanol-acetic acid-water and collidine-ammonia systems (17), filter paper chromatography revealed that the radioactive material isolated from the serum of three patients who had received therapeutic doses of I^{131} moved precisely as did synthetic radiothyroxine. Both the material extracted from serum and synthetic radiothyroxine displayed small amounts of radioactivity with R.F.'s corresponding to those of iodide and diiodotyrosine. Degradation of thyroxine during the chromatographic procedure, leading to the appearance of similar fractions, has been previously described (17).

The recovery from serum of triiodothyronine was not determined. Furthermore the chromatographic systems employed would probably not have permitted resolution of this material from thyroxine. The data, therefore, offer no evidence concerning the extent, if any, to which radioactive triiodothyronine contributed to the observed values of the BEI^{131}.

The concentration of BEI^{131} was determined in the washed erythrocytes of three patients who had received therapeutic doses of radioactive iodine.

TABLE II

Lack of transport of radioactive thyroxine in human erythrocytes

Patient	BEI^{131}	BEI^{131}
	(*% dose/liter plasma*)	(*% dose/liter RBC*)
1	.301	.000
2	.203	.000
3	.214	.002

TABLE III—*Summary of values obtained in diverse states of thyroidal function*

Patient	Age	Sex	24-Hour BEI^{131} (% dose/liter)	72-Hour BEI^{131} (% dose/liter)	24-Hour thyroidal I^{131} uptake (% dose)	24–72 Hour change in I^{131} uptake* (% dose)	BMR (% normal)
				Euthyroidism			
1	59	F	.009	.062	41	− 0.8	− 5
2	37	F	.012	.036	33		
3	35	M	.016	.035	37	− 0.4	−16
4	42	F	.014	.038	29	+ 1.1	+ 4
5	29	F	.008	.023	25	+ 8.6	− 8
6	43	F	.011	.033	19	+ 4.9	−21
7	35	F	.020	.063	28	− 1.9	
8	38	F	.006	.020	30	+ 4.6	−20
9	27	F	.014	.043	40	+ 2.3; −2.5	−10
10	26	F	.020	.047	39	+ 2.1	− 2
11	29	F	.022	.046	26	+ 2.0; −1.6	−17
12	58	F	.010	.030	34	− 1.3	−29
13	25	F	.010	.028	29	− 0.2	+ 3
14	37	F	.012	.044	35	+ 2.2	−20
15	29	M	.008	.022	17	+ 0.7	+ 8
16	59	M	.018	.040	36	+ 2.2; −1.9	+22
17	51	F	.018	.032	23	+ 0.5	+ 4
18	41	F	.032	.078	40	+ 1.0; −1.3	+ 3
19	37	F	.017	.051	32	+ 2.2	−26
20	27	F	.045	.079	30	+ 3.6; −3.3	+ 2
21	47	F	.019	.039	20	− 0.6	+ 6
22	41	F	.013	.032	32	+ 0.4; −0.2	
23	28	F	.023	.028	20	+ 2.3; −1.5	− 5
24	55	F	.018	.040	20	+ 1.9	
25	40	F	.028	.050	21	+ 0.1	− 5
26	38	F	.020	.057	43	− 5.4	−28
27	44	M	.010	.038	24	+ 1.9; −2.2	−33
28	52	M	.012	.017	29	+ 1.4	−10
29	44	F	.005	.041	28	+ 0.8; −1.0	
30	33	F	.010	.033	31	+ 4.0; −2.0	+ 6
31	32	F	.014	.054	30	− 1.0	−11
32	43	F	.030	.053	32	− 0.6	−18
33	58	M	.004	.015	37	+ 7.8	−30
34	27	F	.014	.043	37	+ 3.2; −1.3	+ 7
35	57	F	.005	.016	32	+ 3.4; −1.5	
36	32	F	.016	.015	32	+ 2.0	− 5
37	35	F	.010	.053	44	+ 0.5; −0.7	+15
38	31	F	.022	.036	39	+ 2.0; −0.7	+19
39	49	F	.008	.020	14	+ 0.5; −0.2	− 4
40	50	F	.041	.067	49	+ 0.6; −0.3	−30
41	33	F	.025	.067	32	− 0.5	−13
42	55	F	.012	.051	39	+ 0.7	
43	27	F	.013	.020	57	+ 1.8	− 4
44	30	F	.008	.030	29	+ 0.9	−14
45	71	F	.018	.021	18	+ 1.9	− 6
46	48	F	.014	.021	34	+ 2.5; −1.5	
47	30	M	.008	.019	18	+ 0.2; −1.2	−24
48	33	M	.022	.042	25	+ 2.0	−23
49	48	M	.010	.062	26	+ 6.0; −4.0	−21
50	36	F	.007	.018	13	− 1.2	− 6
51	15	F	.033	.044	24	+ 2.3	− 9
52	38	F	.017	.035	34	− 5.4	−29
53	31	F	.014	.020	55		+ 2
54	62	F	.010	.024	27	− 0.2	− 8
55	33	F	.012	.038	26	+ 0.6	+ 4
56	38	F	.010	.022	17	+ 3.4	+ 4
57	28	F	.012	.014	40	− 0.8	
58	32	F	.016	.035	23		0
59	28	F	.018	.018	16		
60	28	M	.020	.039	18		+ 5
61	32	F	.014	.041	44	+ 5.3; −2.0	+13
62	35	M	.009	.024	26		
63	30	M	.015	.052	25		−10
64	28	M	.012	.032	21		−12
		Mean	.016	.037	30	+ 0.1	
		Std. Dev.	.008	.016	9	2.6	

* Two figures given in patients demonstrating Pattern No. 2 (see Text). The negative value in these cases is used in calculations.

TABLE III—*Continued*

Patient	Age	Sex	24-Hour BEI^{131} (% dose/liter)	72-Hour BEI^{131} (% dose/liter)	24-Hour thyroidal I^{131} uptake (% dose)	24–72 Hour change in I^{131} uptake* (% dose)	BMR (% normal)
Hyperthyroidism							
65	37	F	.747	1.085	80	−18.5	+50
66	22	M	.018	.023	65	− 1.2	+37
67	46	F	.151	.338	94	−22.1	+41
68	29	M	.036	.204	76	− 6.7	+35
69	22	F	.568	.712	96	− 5.6	+50
70	28	F	.019	.250	38	− 3.2	+37
71	44	F	.053	.127	77	− 4.9	+42
72	42	F	.085	.150	97	− 4.4	+55
73	60	M	.141	.308	66	+ 9.3; −4.1	
74	53	F	.198	.468	65	+ 2.0; −2.7	+28
75	58	F	.202	.549	75	−25.8	+37
76	29	M	.034	.253	57	− 4.7	+49
77	28	M	.104	.283	95	− 9.7	+40
78	51	F	.230	.548	69	−16.3	+72
79	30	F	.104	.429	48	+10.4; −2.0	+30
80	41	F	.081	.327	56	+ 0.2; −2.0	+33
81	37	F	.054	.163	55	+ 6.0; −2.5	+30
82	35	F	.850	1.170	76	− 6.0	+62
83	27	F	.488	1.040	94		
84	37	F	.340	.862	76	−12.3	+55
85	28	F	.019	.079	42	− 0.4	+18
86	23	M	.047	.080	56	+ 3.3	+30
87	41	F	.166	.301	80	− 3.2	+42
88	59	F	.020	.081	45		+25
89	62	F	.359	.611	82		+38
		Mean	.205	.418	70	− 7.0	
		Std. Dev.	.232	.104	18	7.2	
Thyroidal Inactivity							
90†	32	F	.002	.002	2		−38
91‡	23	M	.003	.004	19		−28
92‡	24	M	.004	.006	17		−30
93†	28	F	.004	.003	10		−35
94†	14	F	.006	.008	2		−30
95§	35	F	.002	.006	1		
96§	36	M	.010	.011	7		+15
97†	45	F	.003	.002	1		−38
98†	50	F	.004	.004	2		−40
99†	28	F	.006	.007	2		−36
100‡	47	M	.006	.006	4		−25
101‡	55	F	.003	.008	4		−27
102†	30	F	.006	.005	3		−45
103§	36	M	.004	.008	9		
		Mean	.004	.006	6		
		Std. Dev.	.002	.003	6		
Post-Thyroidectomy ‖							
104	24	F	.318	.066	7		−38
105	8	F	.224	.159	4		−30
105a¶	8	F	.162	.040	4		
106	29	M	.121	.078	3		−35
106a	29	M	.090	.083	4		
107	18	F	.088	.025	2		−37
108	28	M	.010	.037	2		−29
108a	28	M	.060	.048	9		
109	40	F	.072	.080	15		−25
109a	40	F	.069	.086	21		
		Mean	.121	.070	7		
		Std. Dev.	.091	.038	6		

† Primary myxedema.
‡ Hypopituitarism.
§ Euthyroid receiving exogenous thyroid.
‖ All patients in this group underwent thyroidectomy for thyroidal carcinoma.
¶ Repeat test in same patient.

Although simultaneously determined concentrations of BEI^{131} in the plasma were high, the radioactivity extracted from the erythrocytes was indistinguishable from background radiation (Table II).

In all euthyroid and hyperthyroid patients, the concentration of BEI^{131} in the serum increased during the interval between 24 and 72 hours after the administration of the tracer (Table III). Patients with decreased thyroidal function associated with primary or pituitary myxedema, or with the ingestion of thyroid substance shall hereafter be referred to as patients with thyroidal inactivity. In this group of cases, significant differences between the 24 and 72 hour concentrations could not be detected. In five of six patients with carcinoma of the thyroid in whom total thyroidectomy had been attempted, and in two patients with thyrotoxicosis who continued to take propylthiouracil throughout the period of observation (not shown in Table III) the 24-hour concentration of BEI^{131} exceeded that found at 72 hours.

The mean values of the BEI^{131} concentrations in patients with thyrotoxicosis, euthyroidism, or thyroidal inactivity differed significantly from that found in the other two groups ($p < .01$), both at the 24-hour and at the 72-hour period. However, a better separation between the values found in patients with thyroidal inactivity, euthyroidism, and hyperthyroidism was found at the 72-hour period (Figure 1). In only one patient presumed to have thyrotoxicosis did the 72-hour concentration of BEI^{131} fall definitely within the normal range. This patient (No. 66) was a 22-year old male with severe exophthalmos, goiter, a thyroidal radioiodine uptake of 65 per cent at 24 hours, and very mild symptoms of thyrotoxicosis. In three patients in whom the diagnosis of thyrotoxicosis had been made, the BEI^{131} values were just above the range of normal. Of these patients one (No. 86) was a young male with exophthalmos, goiter, a 56 per cent radioiodine uptake, and minimal symptoms of thyrotoxicosis. The other two patients (No. 85, No. 88) were anxious women in whom the diagnosis of thyrotoxicosis was in doubt at the outset.

In one patient (No. 96) with thyroidal inactivity, the BEI^{131} value fell at the lower end of the normal range. This patient was a 36-year old male with non-toxic nodular goiter, who was receiving 2 gr. of dessicated thyroid substance daily, and whose 24-hour radioiodine uptake was 7 per cent.

In hyperthyroid patients, the mean net change in thyroidal uptake between 24 and 72-hours (−7.0 per cent) was significantly different ($p < .01$) from that found in euthyroid subjects (+0.1 per cent). This was also true when the net change was calculated in each patient as percentage of the 24-hour radioactive iodine uptake. (−9.0 per cent *vs.* +0.6 per cent). Successive determinations of thyroidal uptake at 24, 48, and 72 hours disclosed three patterns. The first was a progressive increase in the thyroidal content of radioiodine, the second an increase followed by a decrease, and the third a successive decrease. Of the euthyroid patients, 41 per cent demonstrated pattern 1, 36 per cent, pattern 2, and 23 per cent pattern 3. Of the hyperthyroid patients, 4.6 per cent (one patient) demonstrated pattern 1, 18.2 per cent pattern 2, and 77.2 per cent pattern 3. The severity of thyrotoxicosis could not be consistently correlated with the pattern of consecutive neck counts. However, those patients with the most severe thyrotoxicosis generally demonstrated pattern 3, while those with the mildest disease displayed either pattern 1 or a small net loss of radioactivity of the type found in pattern 2.

In patients with decreased thyroidal function, the low level of radioactivity in the neck precluded the accurate measurement of progressive changes

TABLE IV

Correlation between the functions studied in various clinical groups

Functions	r	p
24 Hr. BEI^{131} *vs.* 72 Hr. BEI^{131}		
Euthyroid	0.59	<.001
Hyperthyroid	0.91	<.001
Combined*	0.63	<.001
72 Hr. BEI^{131} *vs.* 24 Hr. thyroidal uptake		
Euthyroid	0.23	>.05
Hyperthyroid	0.35	>.05
Combined	0.66	<.001
72 Hr. BEI^{131} *vs.* "Net change"†		
Euthyroid	−0.34	<.01
Hyperthyroid	−0.44	<.02
Combined	−0.40	<.001
24 Hr. thyroidal uptake *vs.* "Net change"		
Euthyroid	−0.06	>.05
Hyperthyroid	−0.20	>.05
Combined	−0.61	<.001

* Euthyroid and hyperthyroid groups considered together.

† 24–72 Hour change in thyroidal I^{131} uptake.

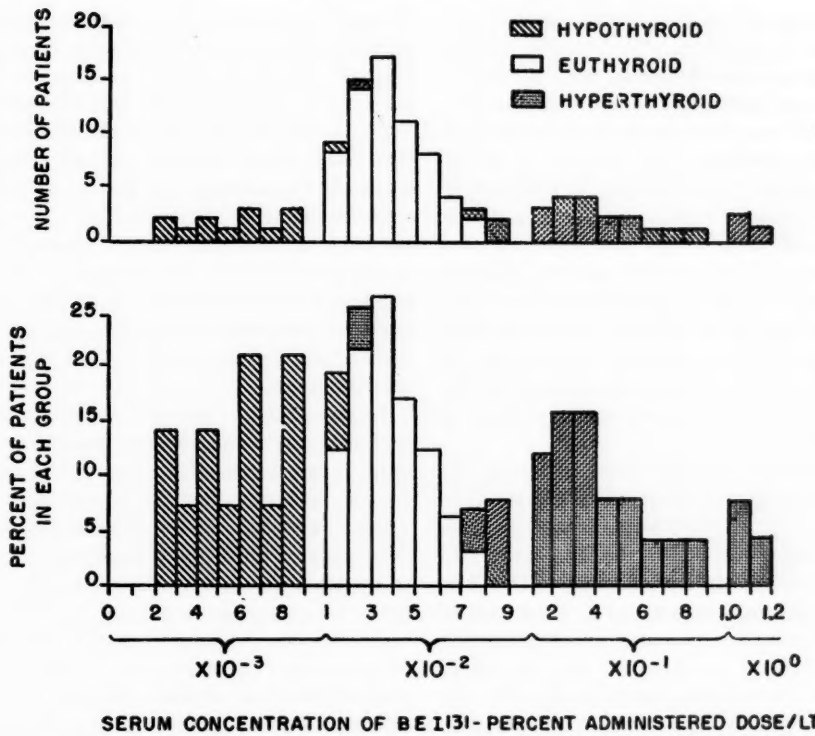

FIG. 1. DISTRIBUTION OF 72-HOUR CONCENTRATIONS OF BUTANOL-EXTRACTABLE I^{131} IN THE SERUM OF HYPERTHYROID, EUTHYROID, AND HYPOTHYROID PATIENTS (EXCLUDING PATIENTS WITH THYROIDAL CARCINOMA)

in the uptake of radioactive iodine. Nevertheless, it appeared certain that some thyroidal accumulation and storage of I^{131} had occurred, since measureable radioactivity persisted in the region of the thyroid gland for several days.

Statistical correlations between the various functions studied are presented in Table IV.

DISCUSSION

Recovery experiments have demonstrated that the extraction procedure described is relatively specific for the principal iodine-containing compound in the plasma, thyroxine. In confirmation of previous reports, thyroxine has been found to be completely absent from the erythrocyte (18).

Measurement of the concentration of radioactive thyroxine in the serum following the administration of a standard dose of radioiodine has been found to be a rather reliable guide to the state of thyroidal function in patients with thyrotoxicosis, euthyroidism, and thyroidal inactivity. In several patients who had undergone virtually complete thyroidectomy, however, the concentration of BEI^{131} did not correlate well with the clinical status of the patient. An explanation for this discrepancy was sought in an analysis of the factors determining the concentration of BEI^{131} in the plasma.

The principal and perhaps only source of plasma's thyroxine appears to be the pool of hormonal iodine within the thyroid gland. Therefore, the relationship described by Zilversmit, Entenman, and Fishler for a product substance and its immediate precursor can be applied to the relationship existing between thyroxine in the plasma and in the thyroid gland (19). These authors have demonstrated that when the precursor, and thence the product, is labelled with a radioactive component, the specific radioactivity (radioactive substance

X/total substance X) of the precursor initially exceeds that of the product. The specific activity (S.A.) of the product then rises with a rapidity which is, at any time, proportional to the magnitude of the difference between the S.A.'s of the precursor and the product. The peak S. A. of the product is reached when the specific activities of the two have become equal. As the S.A. of the product exceeds that of the precursor, its own S.A. begins to decline. If it be assumed that the content of non-radioactive hormonal iodine in the thyroid gland and in the plasma remain constant during the period of observation, then changes in the radioactivity of the thyroid gland or of the plasma's BEI will be proportional to changes in their respective S.A.'s.

The concentration of hormonal iodine in the plasma of hyperthyroid patients is greater than normal (20, 21). Nevertheless, in such patients following a tracer dose of I^{131}, the S.A. of the PBI increases more rapidly than normal (22). The present findings suggest that the rate of increase of the S.A. of the BEI is also augmented in patients with thyrotoxicosis. In such patients, the S.A. of the glandular hormonal iodine must therefore exceed, at least initially, that found in patients with normal thyroidal function. The relatively greater S.A. of the thyrotoxic gland commonly results from an increase in the initial accumulation of I^{131} and from a reduction in the total quantity of hormone stored within the gland (23).

The "net change" in glandular radioactivity describes the direction and magnitude of changes in the S.A. of organically-bound iodine within the gland during the 24 to 72-hour interval. A decrease in the S.A. of the glandular hormone [3] was seen more often in thyrotoxic than in normal patients. The average thyroidal S.A. increased by 0.6 per cent in normal patients, but it decreased by 9 per cent in thyrotoxic patients. The more rapid loss of radioactivity from the thyroid glands of thyrotoxic patients results from an increase in the rapidity of disappearance of radioiodide from the plasma (24) which hastens the cessation of collection of radioiodine, and from an increase in the rate of turnover of glandular hormone, as will be seen below.

Thus in thyrotoxic patients, the S.A. of the plasma's hormone is increasing and that of the glandular hormone is decreasing more rapidly than normal. The time interval required for the two to become equal and for the concentration of BEI^{131} to reach a peak, should therefore be shorter than normal in thyrotoxic patients. This proves to be the case when re-utilization of radioiodide made available by the peripheral degradation of hormone is prevented (22). However, when as in the present study, re-utilization of radioiodide is allowed to occur, the decline in the S.A. of the glandular hormone is retarded, the peak concentration of the plasma's BEI^{131} is delayed, and its level increased.

In the patients "totally" thyroidectomized for thyroidal carcinoma, the concentration of BEI^{131} in the serum decreased during the interval between 24 and 72 hours after the administration of the tracer. The S.A. of the BEI in the plasma of these patients must, therefore, have exceeded that of the glandular hormone more quickly than normal. This could be explained by either a more rapid decrease in the S.A. of the glandular hormone, a more rapid increase in the S.A. of the plasma's hormone, or both. All patients who demonstrated this pattern were hypothyroid, and in three the PBI was found to be subnormal. Since these patients demonstrated a normal or increased concentration of BEI^{131} in their serum at 24 hours, it may be concluded that the S.A. of their circulating hormonal iodine had increased more rapidly than normal. However, the presence in the plasma of normal or increased concentrations of BEI^{131} suggests that rather large amounts of radioactive hormone had been released. Furthermore, the hypothyroid state of these patients indicates that the total amount of hormone released by their thyroid tissue per unit time was subnormal. Thus the S.A. of the hormone released by these glands must have been greater than normal. This conclusion, together with the markedly decreased initial uptake of I^{131} found in the thyroid glands of these patients, indicates that the radioactive hormone formed was minimally "diluted" by preformed, non-radioactive hormone. The total amount of hormone stored by these thyroid glands must, therefore, have been considerably decreased.

A similar decrease in the concentration of BEI^{131} between 24 and 72 hours after the administration of the dose was noted in the serum of two pa-

[3] The term "hormone," used in this sense, refers to all iodinated tyrosyl compounds within the gland.

tients with thyrotoxicosis made euthyroid by the administration of propylthiouracil. This agent reduces the rate of formation of thyroid hormone (25, 26) but does not diminish, and may rather increase the rate of release of hormone from the gland (27). In the hyperthyroid patient, this must result in a marked depletion of glandular hormone.

It is apparent that the concentration of BEI^{131} in the serum does not always accurately reflect the overall rate of hormone formation or release. For any given rate of radioiodine accumulation, depletion of glandular hormone results in an increased release of BEI^{131}. To the extent that depletion of glandular hormone occurs in patients with thyrotoxicosis, the sensitivity of the test in detecting the hyperthyroid state is increased. However, when depletion of glandular hormone occurs in patients who are not thyrotoxic, the concentration of BEI^{131} in the serum does not correlate well with the clinical state. Two cases of postoperative myxedema demonstrating this phenomenon have recently been reported (28).

It is possible to evaluate further the manner in which the amount of glandular hormone influences the concentration of BEI^{131} in the serum. It will be assumed that the maximal accumulation of radioiodine within the thyroid gland is instantaneous, and that within the first three days after the administration of the tracer, peripheral utilization of released radioactive hormone is negligible.[4]

If A = thyroidal content of I^{131} at time, t (per cent of administered dose).
A_0 = the peak thyroidal accumulation of I^{131} (per cent of administered dose).
(BEI^{131}) = concentration of BEI^{131} in the serum at time, t (per cent dose/liter serum).
V = volume of distribution of BEI^{131} (total extrathyroidal $BEI^{131}/(BEI^{131})$.
r = rate of turnover of glandular hormone (per cent of glandular hormone synthesized or released per day).
t = time after peak thyroidal accumulation of I^{131} (days).

The amount of I^{131} within the thyroid gland at any time,

$$A = A_0 e^{-rt}$$

The rate of release of radioactive hormone,

$$\frac{d(BEI^{131})V}{dt} = rA_0 e^{-rt}$$

Therefore, the total amount of BEI^{131} released up to time, t,

$$(BEI^{131})V = -A_0 e^{-rt} + C$$

Since when $t = 0$, $(BEI^{131})\ V = 0$,

$$C = A_0$$

and

$$(BEI^{131})V = A_0(1 - e^{-rt}) \qquad (1)$$

At any time, and for any value of V and A_0, the concentration of BEI^{131} in the serum will depend upon the rate of turnover of glandular hormone (r). Therefore, at any given rate of hormone manufacture, decreases in the quantity of glandular hormone increase the concentration of BEI^{131} in the serum by increasing the rate of turnover of hormone within the thyroid gland.

Unlike the thyroid tissue of patients designated as having thyroidal inactivity, that of the patients who had been thyroidectomized is characterized by a high rate of turnover of glandular hormone. It is not known whether, in these patients the remaining thyroid tissue is normal or neoplastic in character. If it were the latter, then the increased turnover rate could be the result of an inability of the tissue to store iodine because of a structural characteristic, such as absence of follicles (29). It is more likely, however, that the increased turnover rates reflect an intense stimulation of the residual thyroid tissue by thyrotropin (30). In the latter instance, the failure of the residual tissue to maintain a euthyroid state could result solely from an inadequate glandular mass. In either case, attempts to alter the function of these thyroidal remnants in such a manner as to make possible therapy with radioactive iodine might well be directed towards increasing the thyroidal storage

[4] It is, of course, recognized that the maximal thyroidal accumulation of I^{131} is not instantaneous. Furthermore, it is likely that considerable utilization of radioactive hormone occurs during the 72-hour period of observation, especially in patients with thyrotoxicosis. Nevertheless, the mathematical relationships defined are considered to be first approximations to those which truly pertain to the functions under consideration.

of hormone as well as increasing the avidity for iodine.

Equation 1 suggests that it may be possible to evaluate the rate of turnover of glandular hormone by examining the relationship between the BEI^{131} and the initial accumulation of radioiodine within the thyroid gland. From equation 1, the slope of the curve representing the relationship between these two functions may be described as

$$\frac{d(BEI^{131})}{dA_0} = \frac{(1 - e^{-rt})}{V} \qquad (2)$$

For any value of t, the slope will be a straight line, provided that both V and r are constant.

Figure 2 illustrates the relationship observed in the present study between the concentration of BEI^{131} in the serum 72 hours after the administration of the tracer and the 24-hour thyroidal uptake. The values displayed by patients with thyroidal carcinoma, although shown on the chart, are not included in the calculations made from this figure. In each of the other patients, the observed 24-hour thyroidal uptake of radioiodine is "corrected" for the amount of radioactive hormone which has been released by this time. This correction is made by adding to the observed uptake the product of the 24-hour concentration of BEI^{131} in the serum and an assumed volume of distribution of BEI^{131} of 24 liters (27). The curve shown is the visual best fit to a series of points representing the average value of all patients within each 10 per cent uptake-interval. Rather than following a straight line, the slope of the curve rises sharply as the uptake approaches 100 per cent. Since the volume of distribution of thyroid hormone apparently does not differ among patients with varying states of thyroidal function (27), equation 2 indicates that as the maximal uptake increases, the rate of turnover of glandular hormone increases. This conclusion is in accord with direct measurements, which reveal an increased rate of release of hormone from thyrotoxic glands (31).

The curve shown in Figure 2 is described by the equation

$$\text{Uptake} = \frac{100(BEI^{131})}{.09 + (BEI^{131})} \qquad (3)$$

However, the relationship between the thyroidal rate of clearance of plasma iodide and the uptake of radioactive iodine has been described previously by the following equation (32):

$$\text{Uptake} = \frac{100 \times \text{thyroidal iodide clearance } (C_t)}{\text{thyroidal clearance } (C_t) + \text{renal clearance } (C_r)} \qquad (4)$$

Therefore, from equations 3 and 4,

$$\text{Uptake} = \frac{100(BEI^{131})}{.09 + (BEI^{131})} = \frac{100C_t}{C_t + C_r} \qquad (5)$$

$$(BEI^{131}) = \frac{.09\,C_t}{C_r} \qquad (6)$$

If the average normal rate of renal clearance of iodide is assumed to be 2.0 liters per hour (33),

$$(BEI^{131}) = .045\,C_t \qquad (7)$$

where C_t = thyroidal iodide clearance rate (liters per hour).

The BEI^{131} concentration in the serum of patients with hyperthyroidism, euthyroidism, and thyroidal inactivity, therefore, appears to be directly proportional to the thyroidal rate of clearance of plasma radioiodide. This conclusion is in accord with the *a priori* assumption that the thyroidal rate of accumulation of iodide is equal to the rate of release of hormonal iodine. Employing equation 7, and the average normal value for the BEI^{131} found in the present study, a thyroidal clearance rate of 0.78 liters per hour may be derived, which is in accord with direct measurements of this function (33–35). From equation 1, together with equations 3, 4, and 7, it is possible to estimate the average rate of turnover of thyroidal hormone for any value of the thyroidal clearance rate, the 72-hour BEI^{131}, or thyroidal uptake of radioactive iodine. It can be shown (see Appendix) that

$$-r = \frac{\ln (.9784 - .0108\,C_t)}{3} \qquad (8)$$

$$-r = \frac{\ln (.9784 - .24(BEI^{131})}{3} \qquad (9)$$

$$-r = \frac{\ln \left(1 - \frac{2.16}{100 - A_0}\right)}{3} \qquad (10)$$

From equation 9, the turnover rate associated with the average normal value of the BEI^{131} found in the present study is approximately 1 per cent per day. There is considerable evidence that the aver-

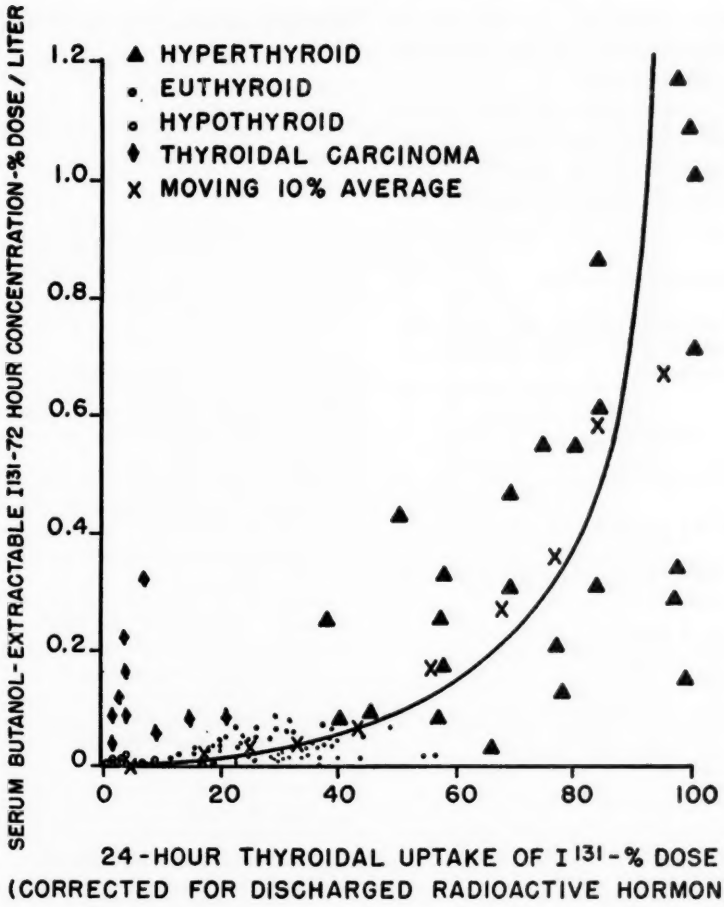

FIG. 2. THE RELATIONSHIP BETWEEN 24-HOUR THYROIDAL UPTAKES OF I^{131} AND 72-HOUR CONCENTRATIONS OF BUTANOL-EXTRACTABLE I^{131} IN SERUM

Thyroidal uptakes have been "corrected" by adding to the observed uptake the quantity of radioactive hormone released during this interval (24-hour BEI^{131} × assumed volume of distribution of 24 liters). The curve shown represents the visual best fit to a series of moving averages of 10 per cent-uptake intervals. Values obtained in patients with thyroidal carcinoma have been excluded from calculations of interval-averages.

age normal daily production of thyroxine is approximately 70 mcg. daily (*27*). The hormone content of the average normal thyroid gland can, therefore, be estimated to be approximately 7000 mcg., which value agrees closely with those obtained by direct chemical analysis (36, 37).

SUMMARY

1. A method for the determination of the BEI^{131} of serum has been shown to measure principally the radioactivity of circulating thyroxine, and has been applied to the study of the concentration of BEI^{131} in the serum of 109 patients with various states of thyroidal function.

2. These results have been correlated with the 24-hour thyroidal uptake of radioiodine and with the subsequent change in thyroidal radioiodine content during the 24–72 hour period.

3. Analysis of the results reveals that the concentration of BEI^{131} in the serum is dependent on

the initial thyroidal uptake of I^{131}, the rate of synthesis of thyroid hormone, and the amount of hormone stored within the gland.

4. The 72-hour concentration of BEI^{131} correlates well with the clinical state except in nonthyrotoxic patients in whom the quantity of hormone stored within the gland is markedly decreased.

ACKNOWLEDGMENTS

The authors are greatly indebted to Mrs. Mary McLaughlin and Maj. Margaret E. Peters, ANC, for invaluable technical assistance.

APPENDIX

From equation 1 (see Text),

$$(BEI^{131})V = A_0(1 - e^{-rt})$$

substituting for (BEI^{131}) and A_0 from equations 4 and 7

$$.045\,C_tV = \frac{100\,C_t}{C_t + C_r}(1 - e^{-rt})$$

where $V = 24$ liters, $C_r = 2$ liters per hour, and $t = 3$ days,

$$1.08\,C_t = \frac{100\,C_t}{C_t + 2}(1 - e^{-3r})$$

$$1.08\,C_t^2 + 2.16\,C_t = 100\,C_t(1 - e^{-3r})$$

$$.0108\,C_t + .0216 = 1 - e^{-3r}$$

$$e^{-3r} = .9784 - .0108\,C_t$$

$$-r = \frac{\ln (.9784 - .0108\,C_t)}{3}$$

Furthermore, from equation 7,

$$C_t = \frac{(BEI^{131})}{.045}$$

Therefore,

$$-r = \frac{\ln .9784 - .24\,(BEI^{131})}{3}$$

According to equation 3,

$$A_0 = \frac{100(BEI^{131})}{.09 + (BEI^{131})}$$

$$.09\,A_0 + A_0(BEI^{131}) = 100(BEI^{131})$$

$$(BEI^{131}) = \frac{.09\,A_0}{100 - A_0}$$

Therefore, according to equation 1,

$$\frac{.09\,A_0V}{100 - A_0} = A_0(1 - e^{-3r})$$

$$\frac{.09 \times 24}{100 - A_0} = 1 - e^{-3r}$$

$$\frac{2.16}{100 - A_0} = 1 - e^{-3r}$$

$$e^{-3r} = 1 - \frac{2.16}{100 - A_0}$$

$$-r = \frac{\ln\left(1 - \frac{2.16}{100 - A_0}\right)}{3}$$

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ABNORMALITIES OF COPPER METABOLISM IN WILSON'S DISEASE AND THEIR RELATIONSHIP TO THE AMINOACIDURIA

By A. G. BEARN and H. G. KUNKEL

(From the Hospital of The Rockefeller Institute for Medical Research, New York, N. Y.)

(Submitted for publication September 2, 1953; accepted November 25, 1953)

In recent years an increasing amount of evidence has accumulated suggesting that Wilson's disease is a metabolic disorder associated with disturbances in copper and amino acid metabolism (1–8). The urinary excretion of copper and amino acids are now known to be increased in this disease (2, 3). The total plasma amino acids are not significantly raised (4, 8), and the serum copper concentration, originally considered to be normal or increased, has recently been shown to be decreased (5). A reduced level of serum copper oxidase activity in Wilson's disease has also been briefly reported (5). The findings of Holmberg and Laurell (9) indicated that oxidase activity in the serum is due to ceruloplasmin the major copper containing protein of serum. A deficiency of ceruloplasmin in Wilson's disease, using spectrophotometric and immunological techniques has also been demonstrated (7). A genetic analysis of a large group of patients with Wilson's disease has been reported separately and indicates the disease to be inherited in an autosomal recessive fashion (10).

The experiments to be described in this and a companion paper (11) were undertaken to investigate further the abnormalities in copper and amino acid metabolism in this disease and to correlate these findings with the clinical condition of the patient with emphasis upon the extent of hepatic and neurological involvement. A control group of patients suffering from various types of cirrhosis of the liver was also studied.

MATERIAL AND METHODS

All patients studied were admitted to the hospital. Twenty-four-hour urine samples were collected in acid-cleaned vessels and stored at + 4°C. Thymol crystals or chloroform (6 ml.) and toluene (6 ml.) were added as preservatives. Urinary alpha amino nitrogen was determined by the gasometric ninhydrin method of Van Slyke, MacFadyen, and Hamilton (12). The method of Brand, Harris, and Biloon (13) was used to detect qualitatively the presence of urinary cystine and was also employed as a screening procedure in a search for aminoaciduria in siblings of patients with Wilson's disease. Urinary glucose was estimated using the "Clinitest" [1] reagent, and venous blood sugar according to the method of Nelson (14).

Serum and urinary copper were estimated in duplicate by a slight modification of the method described by Eden and Green (15). Serum copper estimations were carried out in duplicate using 5 ml. samples; for urinary estimation of copper 15 or 20 ml. was normally used. The average difference between duplicate analyses of a sample containing 5 γ of copper was 0.24 per cent. The average difference between duplicates can be calculated from the formula $2/\sqrt{\sigma}$ where σ = S.D. When necessary, copper analyses on 1 ml. samples of serum were carried out with considerable accuracy using the micro modification of the same procedure (15).

Serum copper enzyme (ceruloplasmin) was estimated by the method of Holmberg and Laurell (9) employing paraphenylendiamine (p.p.d.) as substrate. Oxygen uptake was measured in a Warburg flask at 37°C. The solution of p.p.d. containing 5 mg. per ml. was brought to pH 6.0 by the drop-wise addition of 1 N HCl. The pH was measured in a Beckman pH meter. One ml. of the p.p.d. solution together with 1 ml. of acetate buffer of pH 6 was placed in the flask. Five-tenths ml. of serum, dialyzed at + 4°C. against acetate buffer (pH 6.0) for twelve hours, was placed in the sidearm. On all occasions, a control sample using 0.5 ml. of distilled water was used to correct for the oxygen uptake of paraphenylendiamine which occurred in the absence of ceruloplasmin.

Electrophoretic separation of sera was carried out in a starch-supporting medium by a previously described method (16). Special precautions were taken to remove traces of copper by washing the starch with "Versene" [2] and by doubly distilling the water used for the buffer solution and the electrode vessels. The micro-copper procedure (15) was employed for analyses of the serum fractions displaced from the starch segments; 4 ml. of serum were fractionated in each case.

CLINICAL MATERIAL

Seventeen patients with Wilson's disease were studied and compared with 23 patients with cirrhosis and 12 normal subjects.

[1] Ames Company, Inc., Elkhart, Indiana.

[2] The Versene used was kindly supplied by Riker Laboratories, Inc., Los Angeles, California.

TABLE I—*Summary of the clinical and biochemical findings in 16 patients with Wilson's disease*

No.	Patient	Sex	Ethnogenic group	Parental consanguinity	Age at onset	Presenting symptom	Dur. of disease	Present condition	Enlarged liver	ES varices	A/G	Zn turb. units	Bilirubin	BSP % retent. 45 mins.	Glycosuria	Tremor of arms	Rigidity	K-F rings	Urinary copper	Serum copper	Serum copper prot.
					yrs.		*yrs.*				*Gm.*		*mg. %*						*µg./24 hrs.*	*µg./100 ml.*	*µM O_2 uptake/ml./hr.*
1	C. R.	F	Italian	2nd Cousin	21	Ataxia of legs	9	Alive	0	0	4.55/2.30	18	1.4	10	0	++	+	++	688	53	0.594
2	V. R.	M	Italian	2nd Cousin	16	Dysarthria	3	Alive	0	0	4.7/2.9	8	0.9	8	0	+	+++	+	569	63	0.780
3	J. M.	M	Italian	1st Cousin	15	Ataxia of legs	4	Alive bedrid.	0	0	4.4/2.3	19	1.3	10	++	+	+++	+	1065	48	0.348
4	G. V.	M	Negro	1st Cousin	27	Tremor of arms	5	Alive	0	0	4.7/2.8	11	0.5	6	++	++	+	+	875	45	0.866
5	L. A.	M	Jewish	2nd Cousin	32	Tremor of arms	18	Alive	0	0	5.2/1.7	16	0.8	17	+++	++	++	++	1446	77	0.596
6	W. C.	M	Irish	1st half Cousin†	16	Tremor of arms	14	Dead	++	+	3.0/2.75	25	2.0	34	++	+	+	+	453	39	0.225
7	C. P.	M	Czech-Swiss	Nil	20	Tremor of arms	4	Alive	0	0	5.4/3.2	13	0.4	4	0	+++	+	+	320	57	1.000
8	R. G.	M	Jewish	Nil	33	Ataxia of legs	9	Dead	0	0	5.8/2.1	16	0.7	2	+++	++	++	++	1050	54	0.240
9	M. G.	M	Jewish	1st Cousin	28	Tremor of arms	3	Alive	+	0	4.80/2.6	9	0.8	8	0	+++	+	++	644	80	1.37
10	P. C.	F	Jewish	1st Cousin	29	Tremor of arms	5	Alive	0	0	4.8/2.0	9	0.8	15	0	++	+	++	467	100	2.260
11	J. B.	M	Italian	Nil	11	Jaundice ascites	1	Dead	+++	++	1.87/3.74		4.2	40	0	++§	++§	0		77	
12	S. S.	F	Polish	Nil	24	Ataxia of legs	4	Alive‡	0		4.3/2.2	13	0.6	10	0	++	+	+	243	72	0.960
13	T. M.	M	Italian	1st Cousin	12	Abd. pain splenomegaly	6	Alive	+++	++	2.9/1.1	19	1.9	32	0	0	0	+	910	55	1.690
14	M. F.	M	Jewish	Nil	36	Tremor of arms	1	Alive	0	0	5.1/1.5	11	0.6	10	0	+++	+	+	380	62	
15	Y. R.*	F	Jewish	1st Cousin	13	Ataxia of legs	21	Alive	0	0	3.75/3.0	13	0.6	26	0	++	++	+++	623	45	0.884
16	B. R.*	F	Jewish	1st Cousin	13	Nervous grimacing	21	Alive	0	0	4.7/2.1	9	0.4	13	0	++	+	+++	625	43	0.890

* Uniovular twins. † Once removed. ‡ Mental institution. § Onset four weeks before death.

TABLE II

Comparison of serum copper, serum copper enzyme (ceruloplasmin) and urinary copper in normal subjects, patients with cirrhosis of the liver and Wilson's disease *

	Normals [12]	Cirrhosis [23]	Wilson's Disease [17]
Serum copper μg./100 ml.	108 ± 9.8 [11]	171 ± 47 [20]	60 ± 15.4 [17]
Serum "copper enzyme" μM. oxygen uptake/ml./hr.	3.60 ± 0.73 [12]	6.68 ± 2.39 [18]	0.90 ± 0.54 [15]
Urinary copper μg./24 hrs.	48 ± 16.3 [11]	157.4 ± 125.6 [18]	703 ± 318 [16]

[ ] No. of cases

* Mean values with standard deviations are recorded.

The patients with Wilson's disease had varying degrees of hepatic and neurological involvement (Table I). The predominance, in the affected group, of males and of patients of Jewish and Italian extraction, has been discussed in a previous paper (10).

The patients with cirrhosis included 12 suffering from nutritional Laënnec's cirrhosis, 4 with biliary cirrhosis, and a group of 7 young girls with severe liver disease of uncertain etiology which has previously been described (17).

RESULTS

Serum copper

The serum copper levels found in normal subjects, in patients with Wilson's disease and in patients suffering from cirrhosis of the liver are shown in Table II. It will be seen that the mean copper concentration in the serum of patients with Wilson's disease was considerably lower than that found in normal subjects, although in one patient the serum copper concentration was in the normal range.[3] The wide scatter shown for the values obtained for the patients with cirrhosis may be a result of the heterogeneity of the clinical material. The mean serum copper concentration in the three varieties of cirrhosis studied is shown in Table III. The patients with nutritional Laënnec's cirrhosis did not show a serum copper concentration greatly in excess of normal, while those with biliary cirrhosis had high levels. Patients with cirrhosis of the liver of unknown etiology occupied an intermediate position.

[3] An additional case has since been studied and a normal serum copper concentration found.

Serum copper enzyme

The serum copper enzyme (ceruloplasmin) was measured in the three groups studied and the results are illustrated in Table II. A striking decrease in oxidase activity was noted in all patients with Wilson's disease, while an increase in activity was noted in patients with cirrhosis of the liver. Table III illustrates the results obtained when the patients with cirrhosis of the liver are further classified. The greatest activity was again found to occur in the patients with biliary cirrhosis. Those with nutritional cirrhosis had an oxidase activity only slightly greater than normal, and those patients with cirrhosis of unknown etiology again occupied an intermediate position.

A parallelism between the serum copper concentration and the oxidase activity was first shown by Holmberg and Laurell (9). Figure 1 illustrates the parallelism in normal subjects, patients with cirrhosis of the liver and patients with Wilson's disease. The intercept of the regression line drawn to fit the experimental points suggests that in serum some copper is present which is not bound as ceruloplasmin and hence is not enzymatically active. In patients with Wilson's disease the ceruloplasmin activity appears to be somewhat less than would be expected from a consideration of the se-

TABLE III

Comparison of serum copper, serum copper enzyme and urinary copper in various types of cirrhosis of the liver

	Laënnec [10]	*Undetermined etiology [8]	Biliary [5]
Serum copper *μg./100 ml.*	142 ± 8.0 [8]	163 ± 29.1 [8]	245 ± 18.9 [4]
Serum "copper enzyme" *μM oxygen uptake/ml./hr.*	5.46 ± 2.14 [6]	6.22 ± 1.46 [8]	9.45 ± 2.78 [4]
Urinary copper *μg./24 hrs.*	86.2 ± 48.4 [9]	174.7 ± 62.8 [7]	269 565 [2]

[ ] No. of cases.
* Cirrhosis of the liver, occurring chiefly in young girls.

rum copper concentration, indicating that the non-ceruloplasmin copper may be relatively increased.

In an attempt to evaluate the significance of the enzyme activity measured above and to study the distribution of copper among the various serum proteins, analyses of copper and enzyme activity were carried out on fractions of serum separated electrophoretically in a starch supporting medium. Difficulties were encountered because of the inability to prevent completely interference by traces of contaminating copper. In each experiment more copper was found on adding up the values obtained for each electrophoretic segment than was present in the original serum. Most of the contaminating copper became attached to the albumin fraction, a fact that was apparent in repeated experiments with the same serum. The height of the albumin peak was reduced under conditions where extreme care was exerted to exclude contaminating copper. To accomplish this end purified human albumin was applied to the starch block in front of the serum and this albumin picked up copper while the albumin in the serum became less contaminated. Figure 2 illustrates typical results obtained from a large number of sera of the three types tested for copper distribution. The curve obtained for protein in the same electrophoretic segments used for the copper analyses is illustrated for the normal serum. Two major copper peaks are visible, a front peak (dotted) representing the copper at the albumin, and a middle peak in the α_2 globulin region. Determination of oxidase activity in the various fractions showed that all the activity was associated with the α_2 globulin copper peak, which therefore represents ceruloplasmin. In some experiments a blue band against the white starch background could be seen in this region. The serum from the patient with Wilson's disease showed almost complete absence of the α_2 globulin copper peak. In the case of cirrhosis, on the other hand, both this peak and the oxidase activity of the whole serum, were higher than normal.

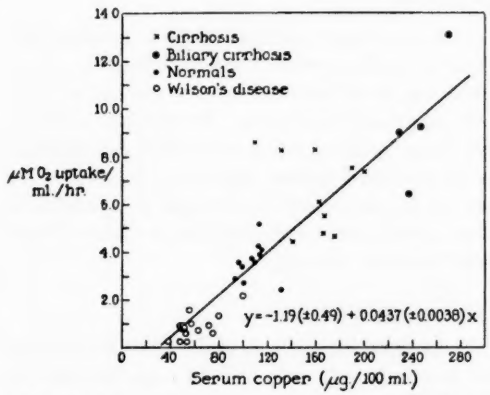

FIG. 1. THE RELATIONSHIP BETWEEN THE SERUM COPPER CONCENTRATION AND SERUM COPPER ENZYME (CERULOPLASMIN)

The significance of the other small peaks is uncertain because of difficulty in defining exactly the baseline. The height of the α_2 globulin copper peak showed very little variation from one experiment to another on the same serum in contrast to the variability of the copper peak in the albumin fraction. In addition, recovery experiments indicated that close to the total copper of the serum from normal individuals and patients with cirrhosis of the liver could be accounted for in this peak. Whether any of the copper found in

the albumin fraction was non-contaminating copper was not clear from these experiments. In the sera of patients with Wilson's disease, the recovery experiments indicated that the low α_2 globulin copper peak accounted for less of the total serum copper than in normal subjects and patients with cirrhosis of the liver. Studies with radioactive copper have shown final localization of radioactivity in the α_2 globulin fraction after oral administration in normal individuals. The latter results confirm the localization of copper in this fraction found in the direct determinations of copper in the electrophoretic fractions.

Urinary copper

Urinary excretion of copper was greatly increased in all patients with Wilson's disease and was slightly increased in most patients with cirrhosis of the liver. These results are illustrated in Tables II and III. Although the highest values were obtained from patients with Wilson's disease, some overlap occurred with patients suffering from biliary cirrhosis. Under standard dietary conditions, day-to-day fluctuations in the urinary excretion of copper in all groups studied were found to be relatively small and to be independent of urinary volume.

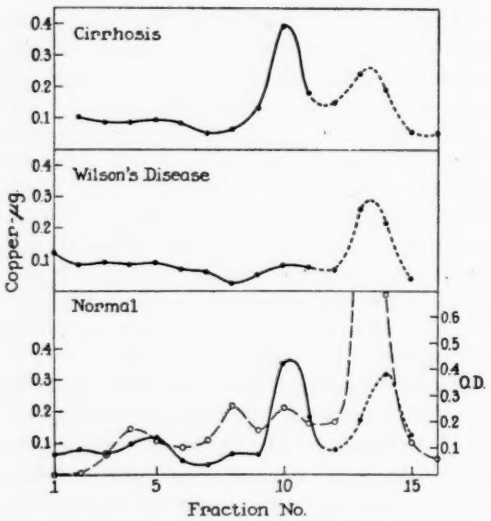

FIG. 2. CURVES SHOWING THE DISTRIBUTION OF COPPER IN VARIOUS SERUM FRACTIONS SEPARATED BY ZONE ELECTROPHORESIS

The protein pattern is illustrated for the normal serum. The ceruloplasmin peak is in fraction 10. The broken line largely represents contaminating copper in the albumin fractions.

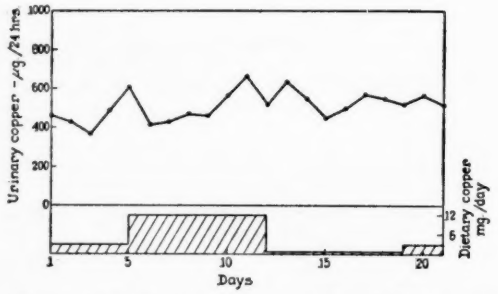

FIG. 3. THE EFFECT OF DIETARY COPPER INTAKE ON THE URINARY EXCRETION OF COPPER IN WILSON'S DISEASE

Amino acids

Urinary excretion of alpha amino nitrogen was increased in 16 of the 17 cases of Wilson's disease. On a fixed protein diet, urinary excretion of alpha amino nitrogen of individual patients with Wilson's disease showed little day-to-day fluctuations, and was independent of urinary volume. Random samples of urine from 35 unaffected siblings of patients with Wilson's disease were examined and no significant aminoaciduria was detected in any of the cases studied, nor was consistent aminoaciduria found in patients with cirrhosis of the liver. Patients in hepatic coma were not tested.

Glycosuria

Glycosuria was found in 5 of the 17 patients with Wilson's disease and in these patients some correlation was noted between urinary glucose and urinary amino acid excretion. In addition, a rise in the amino acid excretion was often accompanied by an increased urinary excretion of glucose. In four of the five cases blood sugar determinations were carried out and showed that the fasting blood sugar was not elevated.

Spectrographic analysis [4]

Spectrographic analysis of the urine was carried out in one patient with Wilson's disease and one

[4] Spectrographic analysis was carried out through the courtesy of Dr. R. O. Roblin, Jr., American Cyanamid Company.

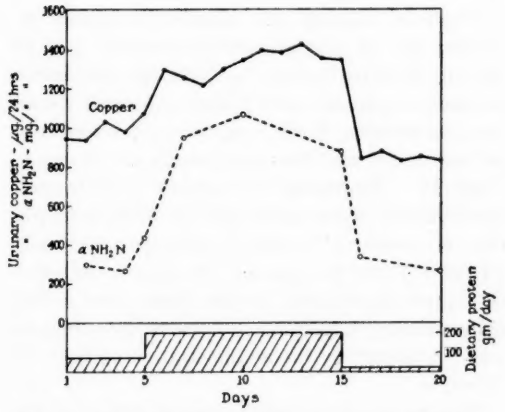

FIG. 4. THE EFFECT OF VARIATIONS IN THE DIETARY PROTEIN ON THE URINARY EXCRETION OF COPPER AND AMINO ACIDS IN A PATIENT (L. A.) WITH WILSON'S DISEASE

normal subject to study the excretion of other trace metals in Wilson's disease. Only the excretion of copper was found to be increased. The levels of iron, cobalt, nickel, and zinc were within normal limits.

Relation of urinary copper and amino acid excretion to dietary intake of copper and protein

Urinary excretion of copper in normal subjects and in patients with Wilson's disease was largely independent of copper intake. Thus, increasing the intake of copper from 0.5 mg. per day to 12 mg. per day did not result in a significant increase

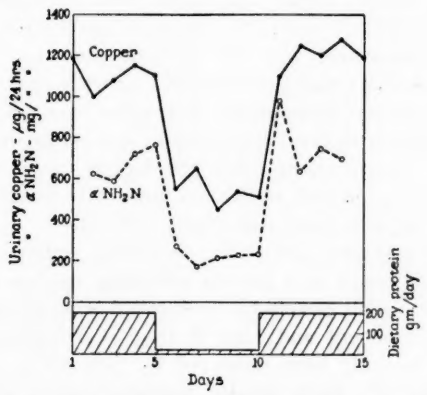

FIG. 5. CHANGES IN THE URINARY COPPER AND AMINO ACID EXCRETION FOLLOWING CHANGES IN DIETARY PROTEIN IN PATIENT (C. P.) WITH WILSON'S DISEASE

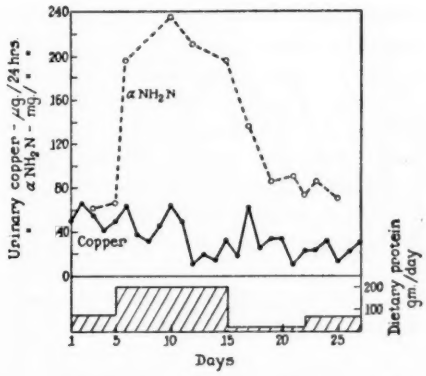

FIG. 6. EFFECT OF DIETARY PROTEIN ON URINARY EXCRETION OF COPPER AND AMINO ACIDS IN A NORMAL SUBJECT

in the urinary excretion of copper (Figure 3). This result was found whether copper in the diet was increased or came from added copper sulfate. In patients with Wilson's disease an increased protein intake resulted in a prompt and parallel increase in the urinary excretion of copper and amino acids. The changes produced in two different patients are illustrated in Figures 4 and 5. In normal subjects increasing the dietary protein caused no detectable rise in the urinary excretion of copper. A typical response is shown in Figure 6.

The excess amino acids excreted in patients with Wilson's disease placed on a high protein diet were greater than occurred in normal subjects on the same diet. Both groups were placed on a 200 Gm. protein diet and the total increased daily excretion of amino nitrogen in Wilson's disease was approximately three times more than occurred in normal subjects.

Effect of cortisone, "Benemid",[8] and mercurial diuretics

The relationship between urinary copper and urinary amino acids in Wilson's disease was further demonstrated by the administration of cortisone. The increase in the amino acid excretion in Wilson's disease which followed the administration of cortisone was again promptly accompanied by a parallel increase in the urinary excretion of copper (Figure 7).

[8] p-[di-n-propylsulphamyl]-benzoic acid.

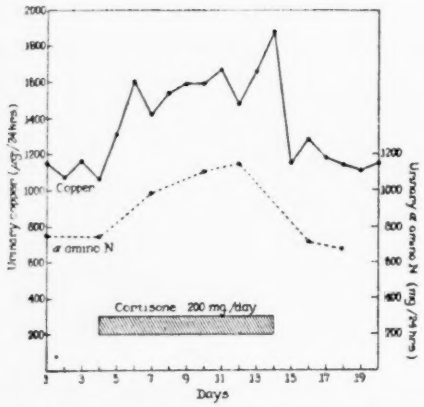

FIG. 7. EFFECT OF CORTISONE ON URINARY EXCRETION OF COPPER AND AMINO ACIDS IN A PATIENT WITH WILSON'S DISEASE

Mercurial diuretics and "Benemid" administered in full doses to two patients were not found to influence the excretion of amino acids in Wilson's disease significantly. Mercurial diuretics had no effect on the urinary excretion of copper. Following the administration of Benemid, however, there appeared to be a slight increase in the urinary excretion of copper in one patient.

Effect of chelating agents

The administration of BAL increased the urinary excretion of copper in all patients to whom it was given (Table IV). This was related to the amount of BAL given since increasing the dose increased the urinary excretion of copper.

TABLE IV *

The effect of various substances on urinary excretion of copper in Wilson's disease

Compound	Dose	No. of patients	Mean increase per 24 hr.
			%
BAL	2.5 mg./Kg.	9	51
Versene (Intravenously)	1 Gm.	1	67
	2 Gm.	3	138
Versene (Orally)	30 mg./Kg.	3	5
Cortisone	100 mg.	3	31
	200 mg.	3	65

* Figures represent mean values obtained during period of maximum effect.

"Versene" (20 per cent solution of calcium disodium salt of ethylenediaminetetracetic acid in 500 ml. of normal saline) was administered intravenously to patients with Wilson's disease. In all cases an increase in the urinary excretion of copper was noted and the mean results are shown in Table IV. Increasing the amount of "Versene" administered again increased the urinary excretion of copper. "Versene" administered orally (Table IV) did not increase the urinary excretion of copper significantly in the doses used. The daily urinary excretion of alpha amino nitrogen was not altered by the administration of BAL or "Versene".

The increased urinary excretion following the administration of BAL or intravenous "Versene" was independent of the degree of hepatic or neurological involvement.

DISCUSSION

The low serum copper concentration in 16 [6] of 17 patients with Wilson's disease confirms earlier work from this laboratory (5). Recently, Lahey, Gubler, Cartwright, and Wintrobe (18) have reported three additional patients with Wilson's disease who had a low concentration of copper in the serum. In patients with cirrhosis of the liver, on the other hand, the serum copper is frequently elevated. It has been shown by van Ravesteyn that copper is normally excreted in the bile (19). This observation is in agreement with the present finding that the degree of elevation of the serum and urinary copper in patients with cirrhosis is due at least in part to the degree of biliary obstruction associated with the cirrhotic process. Patients with classical nutritional Laënnec's cirrhosis without significant biliary obstruction have normal or slightly elevated serum copper and urinary copper concentrations while the higher values encountered in both serum and urine were those occurring in patients with biliary cirrhosis.

The greater part of the circulating serum copper is bound to a protein exhibiting oxidase activity. This protein has been called ceruloplasmin by Holmberg and Laurell (9). Ceruloplasmin has recently been measured by Scheinberg and Gitlin (7) using specific immunochemical techniques and also spectrophotometrically. They re-

[6] See footnote 3.

ported eight patients with Wilson's disease in whom the ceruloplasmin concentration was considerably lower than normal and this finding is in accord with the results of the present study. Holmberg and Laurell (9) were the first to suggest that there was a parallelism between the concentration of copper in the serum and the oxidase activity. Further light on the relation of serum copper to various serum proteins has been derived from the experiments using zone electrophoresis. The major copper peak in normal serum and in the serum from patients with cirrhosis appeared in the α_2 globulin region. The latter peak was markedly depressed in the sera of patients with Wilson's disease. The electrophoretic experiments also confirmed the specificity of the oxidase measurements of whole serum as an index of its ceruloplasmin level. Copper bound to other proteins in serum gave no enzymatic activity with the substrates employed.

The present work does not suggest that the main copper protein in serum (ceruloplasmin) functions as a transport mechanism in serum in a manner similar to the iron binding protein. This possibility was also considered unlikely by Gubler, Lahey, Cartwright, and Wintrobe (20) who have shown that following the oral ingestion of copper there is no change in the "indirect reacting" copper. It is probable that "indirect reacting" copper represents ceruloplasmin. The possibility of an enzymatic function in the body must be considered, particularly in view of the broad distribution of copper enzymes in tissues and the activity exhibited by this protein with synthetic substrates. Whether a deficiency of ceruloplasmin represents the primary genetically determined abnormality in Wilson's disease remains to be determined.

Preliminary observations suggested that there was a parallelism between the excretion of copper and the excretion of amino acids in patients with Wilson's disease (5). This observation was supported and amplified by the recent findings of Matthews, Milne, and Bell (8) who showed that ingestion of amino acids led to a prompt rise in the urinary excretion of copper and by the present work where increases in dietary protein gave rise to an increased excretion of copper and amino acids in the urine. In normal subjects, despite an increase in the urinary alpha amino nitrogen, there was no detectable increase in the urinary copper excretion. In Wilson's disease as a result of a high protein diet an increased absorption of amino acids will occur and some of the excess amino acids may combine directly with the high tissue copper and an increase in the urinary excretion of copper will result. In normal subjects in whom tissue copper is not raised and in whom the renal threshold is not lowered no significant increase in the urinary excretion of copper will occur.

Although the association between urinary copper and amino acid excretion has been demonstrated there is no evidence that the copper and the amino acids are excreted as copper amino acid complexes, moreover, there are many other substances excreted in the urine other than amino acids to which copper could become bound. An attempt was made to determine the substances in the urine to which the copper was bound by zone electrophoresis experiments following the oral administration of radioactive copper. Several copper peaks were obtained when the experiment was run at the pH of the excreted urine but whether the copper was bound to certain specific amino acids or peptides was not entirely clear.

It has been suggested that in Wilson's disease there is a lowered renal threshold for amino acids and that this abnormality accounts for the finding of an increased excretion of amino acids in the urine (4, 8). The present finding that, following the administration of a high protein diet to patients with Wilson's disease, a greater quantity of amino acids was excreted in the urine than occurred in normal subjects could also be cited to support this hypothesis. However, consideration of the behaviour of individual amino acids, which is discussed more fully in the following paper (11), suggests that such a hypothesis may not explain all of the experimental findings.

The increased excretion of copper following cortisone was expected since it is known that cortisone increases the urinary excretion of amino acids (21). Mercurial diuretics and "Benemid," substances well known to have an action on the renal tubules, did not alter amino acid excretion and hence urinary copper excretion was not significantly increased. The increased urinary excretion of copper following the administration of BAL, or "Versene" intravenously was not associated with an increase in amino acid excretion, probably because both BAL and "Versene" are

themselves substances which form a chelate with copper which is readily excreted in the urine.

"Versene" administered orally did not result in an increase in the urinary excretion of copper in patients with Wilson's disease. This observation is in contrast to recent findings in the treatment of lead poisoning (22) where an increased urinary excretion of lead followed the administration of "Versene" orally. The dose employed in the treatment of lead poisoning, however, was twice that employed in the present study.

Van Ravesteyn (19) has suggested that in normal subjects urinary copper excretion does not reflect dietary intake of copper. This study furnishes further evidence for this concept, since both normal subjects and patients with Wilson's disease, provided they were placed on a constant protein intake, did not show a significant increase in the urinary excretion of copper despite very large variations in copper in the diet. However, following the intravenous administration of trace amounts of radioactive copper it has been possible to demonstrate an increased urinary excretion of copper and a decreased fecal excretion compared with normal subjects.

There was no clear cut relation between the duration or severity of the disease process in patients with Wilson's disease and the degree of abnormality found in copper and amino acid metabolism. These biochemical findings were similarly unrelated to the degree of hepatic or neurological involvement or the presence of Kayser-Fleischer rings. The clinical improvement which sometimes appeared to follow the administration of various chelating agents was not related to the amount of copper excreted; the latter was similar in all types of the disease whether or not subsequent improvement occurred.

The finding that Wilson's disease is inherited in an autosomal recessive fashion (10) suggested the possibility that asymptomatic siblings might possess a biochemical abnormality. Uzman and Hood (23) have reported a family with Wilson's disease in whom examination of four asymptomatic siblings revealed an aminoaciduria. However, this family, in view of the large number of biochemically affected individuals described, is genetically atypical. It is also noteworthy that examination of 35 siblings in the present study failed to reveal an aminoaciduria in any individual. However, an increased amino acid excretion was not found in one patient with undoubted Wilson's disease (11) and this observation suggests that an aminoaciduria may not be an early manifestation of the disease, and examination of asymptomatic siblings for abnormalities in copper metabolism should be preferentially undertaken. Investigation of the copper metabolism in some of the asymptomatic siblings has thus far failed to reveal any abnormalities.

As a hypothesis it is suggested that in Wilson's disease as a result of an increased absorption of copper this metal accumulates in the tissues and appears in the urine. If the deposition of copper in the liver becomes excessive cirrhosis may develop and, in an analogous fashion, accumulation of copper in the kidney may give rise to renal tubular damage. If this occurs an increased urinary excretion of amino acids, peptides and occasionally glucose will result.

SUMMARY

1. Copper and amino acid levels in serum and urine were determined in 17 patients with Wilson's disease and compared with a control group of patients with cirrhosis of the liver as well as with normal individuals.

2. Reduced copper levels in the serum and elevated urinary concentrations of copper were characteristic findings of the group with Wilson's disease. The presence of both abnormalities proved more characteristic of the disease than either one alone.

3. Markedly reduced levels of the serum copper protein, ceruloplasmin, were found in all cases of Wilson's disease, and represented the most specific biochemical abnormality found. The specificity of the enzymatic method for the determination of ceruloplasmin was demonstrated by procedures of zone electrophoresis.

4. A parallelism was demonstrated between the urinary copper and amino acid excretion in Wilson's disease. Increasing the amino acid excretion by increasing the protein intake, or by the administration of cortisone resulted in a parallel increase in the urinary excretion of copper.

ACKNOWLEDGMENTS

It is a pleasure to acknowledge our indebtedness to Doctor Houston Merritt, Doctor Theodore J. C. von

Storch, Doctor Harold Wolff, and Doctor Morris Bender for having so kindly referred patients to us.

We wish to thank Mr. Charles Galati for expert technical assistance.

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THE AMINO ACID CONTENT OF THE BLOOD AND URINE IN WILSON'S DISEASE

By WILLIAM H. STEIN, A. G. BEARN, AND STANFORD MOORE

(From the Laboratories and the Hospital of the Rockefeller Institute for Medical Research, New York, N. Y.)

(Submitted for publication September 2, 1953; accepted November 25, 1953)

Since the report of Uzman and Denny-Brown (1) that Wilson's disease was associated with an aminoaciduria, several studies of this aspect of the disease have appeared. Dent and Harris (2) employed paper chromatograms to demonstrate the generalized nature of the aminoaciduria, while Cooper, Eckhardt, Faloon, and Davidson (3) employed microbiological assay to determine quantitatively the extent of the excretion of eight essential and two non-essential amino acids. Investigations of the blood plasma by these investigators (3) and by Matthews, Milne, and Bell (4), who determined total α-amino nitrogen, have not revealed any abnormalities in the concentration of amino acids in plasma, however. With the development of chromatographic methods employing columns of ion exchange resin, it has become possible to determine quantitatively and simultaneously the concentration of all of the ninhydrin-positive substances in normal and pathological urines (5) and in plasma (6). The application of these methods to a detailed study of the amino acid distribution in the urine and plasma of patients suffering from Wilson's disease forms the subject of this communication.

MATERIALS AND METHODS

Subjects. The subjects studied are described with the same initials and numbers in the preceding paper (7).

Samples of urine and blood. The collection of 24-hour urine specimens has already been described (7). For the collection of the urines from fasting individuals, the subject was instructed to void on arising and reject the specimen, drink about 300 cc. of water, void again after 1 to 2 hours and reject the specimen, drink an additional glass or two of water, and collect the urine during the ensuing 1 to 4-hour interval, the duration of which was recorded. The volume and specific gravity of the specimen were measured, and the urine concentrated *in vacuo* in a rotary evaporator (8) to a sufficiently small volume to raise the specific gravity to about 1.02 to 1.03. Usually, 2 cc. aliquots of samples of urine from patients with Wilson's disease (3 cc. of fasting urine) were taken for analysis. Urine was hydrolyzed in the manner already described (5).

Blood was drawn into heparinized tubes and 10 cc. aliquots of plasma deproteinized with picric acid and prepared for chromatography in the manner described for normal plasma (6).

Chromatographic analyses. The results given in Tables I and II were obtained by chromatographing the urine on 100 cm. columns of Dowex 50 (8 per cent cross-linking) and on 15 cm. columns exactly as already outlined (9). The remaining data on urine (Tables IV and V), and all the data on plasma were obtained by the use of the modified ion exchange method (10) employing 150 cm. columns of 4 per cent cross-linked Dowex 50. In most cases only a single chromatographic analysis was run for each sample of urine or plasma. Identification of the peaks on the urine curve (Figure 1) was accomplished by the use of the three criteria already discussed for the case of normal urine (5).

RESULTS

The amino acid content of urine

A typical elution curve obtained when an aliquot of a 24-hour urine specimen from a patient with Wilson's disease is chromatographed on Dowex 50 is given as the solid line in Figure 1. For comparison, a curve from normal urine taken from previous work (5) is shown as the broken line. The quantitative extent of the aminoaciduria in Wilson's disease can be determined from the data presented in Table I, which indicate that the total daily excretion of most of the amino acids is markedly elevated in many cases. Moreover, two amino acids not present in normal urine in detectable amounts, namely, citrulline and proline, may be found in Wilson's disease. Proline is not listed in Table I because it partially overlaps citrulline with the system shown in Figure 1. This overlap does not interfere with the determination of citrulline for the reason that proline yields a yellow colored compound with ninhydrin which absorbs maximally at 440 mμ and contributes only a negligible amount of absorption at 570 mμ. The citrul-

line color, which absorbs maximally at 570 mμ, almost completely masks the proline compound, however, making it impossible to obtain quantitative data for the latter. The presence of large amounts of proline was readily established when the fractions from the citrulline peak were chromatographed on paper strips. A prominent yellow spot in the proline position was clearly separated from the bluish-brown citrulline spot. Furthermore, when a sample of urine was chromatographed on a column of 4 per cent cross-linked Dowex 50, under conditions where proline and citrulline are well separated, a large proline peak was obtained corresponding to an excretion of about 175 mg. of proline per day.

With the chromatographic system employed for the experiments given in Tables III to VI, citrulline and glycine are not separated and hence are determined together.

Several points of interest emerge when the data in Table I, excluding Patient 2 who will be discussed later, are considered as a whole. The highest levels of excretion relative to the normal are reached by threonine and cystine, which may be elevated twenty-fold. The large amount of threonine in Wilson's disease urine was also noted by Cooper, Eckhardt, Faloon, and Davidson (3). The quantities of serine, glycine, asparagine + glutamine, valine, tyrosine, and lysine may increase 5 to 10 fold, while the amounts of histidine, ornithine, and phenylalanine may rise to 2 to 4 times the normal level. The quantities of amino-

TABLE I

The amino acid content of urine in Wilson's disease *

Amino acid	Patient 1 C. R.	2 V. R.	3 J. M.	4 G. V.	5 L. A.	6 W. C.	Normal range
Glutamine + asparagine †‡	390	130	680	395	710	590	40–100
Aspartic	<10	<10	<10	<10	<10	<10	<10
Glutamic	58	21	100	30	110	87	<10
Glycine	520	220	960	530	900	550	70–200
Alanine + aminoadipic §	230	74	380	220	490	230	20–70
Valine	37	6	55	35	110	63	<10
Leucine	36	15	60	42	65	25	10–25
Isoleucine	26	13	24	37	66	28	10–30
Serine †	275	95	490	285	510	425	25–75
Threonine	440	82	680	490	860	580	15–50
Cystine	220	50	330	220	770	120	<10–20
Methionine	12	5	30	5	5	17	<10
Taurine	85	120	46	140	130	49	85–300
Phenylalanine	50	20	110	60	120	120	10–30
Tyrosine	160	45	380	140	270	420	15–50
Histidine ‖	500	270	650	450	680	580	110–320
1-methylhistidine ‖	47	38	26	140	77	42	50–210
Citrulline	81	10	210	140	440	190	<10
Ornithine ‖	13	5	37	33	130	5	<10
Lysine ‖	200	33	380	230	860	79	10–50
Arginine ‖	10	10	34	10	160	10	<10
Total	3365	1225	5660	4290	7480	4195	800–1200
Urine volume, ml.	1540	930	1380	2220	2300	2830	1000–2000
Total N excreted, Gm.	13.86	—	8.00	12.75	16.70	11.97	
Total α-amino N, mg.							
From columns	393	137	673	416	552	479	
By ninhydrin-CO_2	551	211	842	522	1055	612	100–160

* The amounts excreted are reported in mg. per day. The diets were not controlled.

† In every case the quantity of serine + glutamine + asparagine was determined by chromatograms of the type shown in Figure 1. In one instance serine was separated from glutamine + asparagine by chromatography on 4 per cent cross-linked Dowex 50 and found to be comprised of about 50 per cent of the total on a molar basis. A similar ratio of serine to glutamine + asparagine was found for normal subjects (5). All of the individual values for serine and for glutamine + asparagine are, therefore, calculated on this basis.

‡ Calculated as glutamine.

§ Calculated as alanine. In one case chromatography on 4 per cent cross-linked Dowex 50 (10) revealed the absence of significant quantities of aminoadipic acid.

‖ Minimal values. Quantitative recoveries of the basic amino acids are not obtained in experiments of the type shown in Figure 1.

TABLE II

Amino acid content of acid hydrolysates of urine in Wilson's disease

Amino acid	Amount excreted, mg. per day					
	Total after hydrolysis			Increase after hydrolysis		
	Patient 3	Patient 4	Patient 5	Patient 3	Patient 4	Patient 5
Aspartic	650	510	1135	650	510	1135
Glutamic	1890	1210	—	1790	1180	—
Glycine	1290	975	1810	330	445	910
Alanine *	515	290	820	135	70	330
Valine	115	110	305	60	75	195
Leucine	120	130	250	60	88	185
Isoleucine	65	70	145	41	33	79
Serine	620	410	740	130	125	230
Threonine	620	550	1010	−60	60	150
Cystine	305	285	700	−25	65	−70
Taurine	54	130	135	8	−10	5
Phenylalanine	140	110	235	30	50	115
Tyrosine	420	195	350	40	55	80
Histidine	560	475	720	−90	25	40
1-methylhistidine	30	175	70	4	35	−7
Citrulline	150	121	—	−60	−20	—
Ornithine	85	75	190	48	42	60
Lysine	435	335	965	55	105	105
Arginine		85	65		75	−95
Total	8064	6241		3146	3008	

* Uncorrected for aminoadipic acid.

adipic acid, methionine, isoleucine, leucine, and arginine seem to be the least abnormal. On the average, there appears to be a diminished excretion of taurine and of l-methylhistidine, and separate

TABLE III

Amino acid content of fasting plasma in Wilson's disease

Amino acid	Mg. amino acid per 100 cc. plasma		Normal range
	Patient 9 M.G.	Patient 7 C.P.	
Glutamic acid	0.55	0.65	0.4–1.2
Glycine + citrulline *	2.31	1.55	1.5–2.0
Alanine	2.98	2.20	3.0–3.7
Aminobutyric acid	0.09	0.22	0.2–0.35
Valine	2.05	2.51	2.3–3.7
Leucine	1.48	1.48	1.4–2.3
Isoleucine	0.85	0.73	0.7–1.3
Serine	0.85	0.87	1.0–1.3
Threonine	1.55	1.11	1.2–1.7
Half-cystine	1.57	1.39	1.0–1.3
Methionine	0.44	0.30	0.3–0.4
Taurine	0.31	0.26	0.4–0.8
Proline	2.94	1.64	1.8–3.3
Phenylalanine	1.09	0.98	0.7–1.0
Tyrosine	1.58	0.93	0.8–1.5
Histidine	1.40	0.91	0.8–1.5
Ornithine	0.91	0.78	0.6–0.8
Lysine	2.00	2.65	2.5–3.0
Arginine	0.97	1.07	1.2–2.0
Total	25.9	22.2	25–28

* Calculated as glycine. Assuming a citrulline value of 0.50 mg. per 100 cc., as reported by Archibald (21) for normal fasting plasma, the glycine content would be about 0.20 mg. per 100 cc. less than the figures given.

15 cm. columns run with several urine specimens revealed that 3-methylhistidine (11) was sometimes absent altogether.

Patient 2, a sibling of Patient 1, is unique in that the excretion of most amino acids is in the high normal range. Those amino acids excreted in largest amount, however, are the same that are the most abnormal in the other patients, namely, threonine, cystine, serine, glycine, and asparagine + glutamine. The next highest are alanine, tyrosine, and histidine.

In Patient 5, the total output of amino acids per day is the highest listed. The amount of cystine, lysine, ornithine, and arginine excreted, however, is, relative to the others, the most increased. The levels of these same four amino acids are found to be markedly elevated in cystinuria (12, 13).

The data in Table I show that the total alpha-amino nitrogen accounted for as individual amino acids on the chromatograms is always less than that determined directly by the ninhydrin method of Van Slyke, MacFadyen, and Hamilton (14). The total derived from the column may be expected to be low for several reasons. In the first place, proline, which probably contributes 10 to 20 mg. of alpha-amino nitrogen, is not listed in Table I. In the second place, glutamine is very unstable and over 50 per cent of it is transformed to pyrrolidone

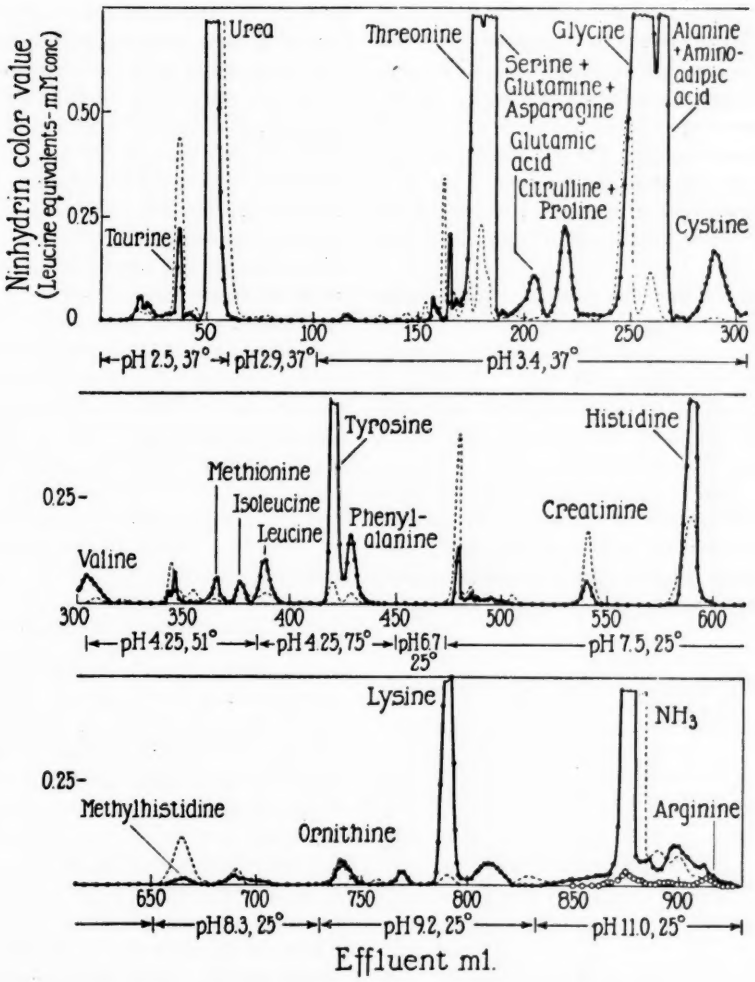

FIG. 1. CHROMATOGRAPHIC SEPARATION OF AMINO ACIDS FROM THE URINE OF A PATIENT WITH WILSON'S DISEASE, PATIENT 3, TABLE I (SOLID LINE)

The column of Dowex 50 (about 8 per cent cross-linking), 0.9 × 100 cm., was operated in the sodium form, with buffers of the pH and temperature indicated as eluants. A sample of 2 cc. of urine was chromatographed. The broken line is the curve obtained previously (5) with normal urine. The crosses in the ornithine peak represent the results obtained by the colorimetric method of Chinard (20). The open circles under the ammonia peak indicate the quantity of ninhydrin-positive material in these alkaline fractions after they had been exposed to reduced pressure to remove ammonia.

carboxylic acid during chromatography and is not determined. Since it can be estimated that at least one half of the glutamine + asparagine peak is actually glutamine, the total quantity of alpha amino nitrogen arising from this source is probably at least half again greater than would be indicated by the figures in Table I. Finally, columns of the type used in these experiments give low recoveries for the basic amino acids, histidine, the methylhistidines, ornithine, lysine, and arginine. The extent of the losses can be estimated roughly from earlier work (cf. [9] Table II).

The values obtained by the ninhydrin method of Van Slyke, MacFadyen, and Hamilton (14) will be too high because cystine evolves about 2 equivalents of CO_2 per amino group and many of the urine specimens are rich in cystine. Taking all these factors into account, the chromatograms appear to account for about 90 per cent of the total alpha amino nitrogen in Patients 1, 3, and 4, 80 per cent in Patients 2 and 6, and 70 per cent in Patient 5.

Like normal urine, the urine of patients with Wilson's disease contains amino acids in conjugated linkages from which the free amino acids are liberated by hydrolysis with acid. A total of about 3 Gm. of amino acids appeared after acid hydrolysis of two urine specimens (Table II), a quantity about double that obtained from normal urine (5). There is thus a smaller proportional elevation in the amount of conjugated amino acids than in the amount of the free amino acids. Certain amino acids, such as serine, threonine, citrulline, cystine, tyrosine, and histidine decompose partially on acid hydrolysis, thus accounting for the loss in some of these amino acids (cf. Table II). The amounts of aspartic and glutamic acids increase the most after hydrolysis, a considerable proportion of the increase doubtless arising as a result of the decomposition of asparagine and glutamine. Conjugated glycine, part of which comes from hippuric acid, despite its great quantity, is at or below normal levels. Taurine, citrulline, histidine, and l-methylhistidine, on the other hand, do not increase in amount after hydrolysis, and hence appear to exist largely in the free form. Conjugated forms of all of the other amino acids seem to exist in urine from patients with Wilson's disease, and in quantities exceeding the normal. The nature, the origin, and the function of many of the conjugated amino acids of urine is not known (cf. [5]). In the investigation of this problem, urine from patients with Wilson's disease may prove to be a useful source of conjugated amino acids.

TABLE IV

Effect of protein intake on the level of urinary amino acids in a single normal subject *

Amino acid	Level of dietary protein		
	15 Gm.	70 Gm.	200 Gm.
Glutamic acid	6	6	7
Aminoadipic acid	5	12	33
Glycine	44	—	98
Alanine	13	—	35
Valine	6	9	20
Leucine	3	6	14
Isoleucine	9	17	41
Serine	25	33	57
Threonine	14	21	34
Half-cystine	9	13	20
Taurine	95	141	163
Proline	<10	<10	<10
Phenylalanine	8	14	17
Tyrosine	16	22	49
Histidine †			
1-methylhistidine	19	91	215
3-methylhistidine	54	67	91
Ornithine	3	3	—
Lysine	6	11	27
Arginine	9	16	9
Total	344†	‡	930†
Urine vol., ml.	520	670	1280
Total N, Gm.	5.1	8.6	23.4

* Excretion given in mg. of amino acids per day.

† Histidine was not determined in these chromatograms because of overlaps with creatinine. The total values will therefore be low by about 100 to 300 mg.

‡ The determinations of glycine and alanine were lost in this case, and hence the total would not be meaningful.

The amino acid content of plasma

The result of the chromatography of a sample of fasting plasma from a patient with Wilson's disease is shown in Figure 2. Qualitatively, the picture appears normal (cf. [6]), an impression confirmed by the quantitative data given in Table III. When compared to the normal range given in the last column of the table, which is based on only five individuals (6), none of the amino acid values is significantly aberrant for both patients; nor do the total quantities of amino acids[1] given at the bottom of the table reveal any noteworthy or consistent trends. Thus, the observation of Cooper, Eckhardt, Faloon, and Davidson (3) based on the ninhydrin method of Van Slyke, MacFadyen, and Hamilton (14) that the total alpha amino nitrogen in fasting plasma in Wilson's disease is normal is confirmed, and supplemented by the finding that the detailed amino acid distribution is also normal.

The effect of diet upon the levels of amino acids in urine and plasma

There is evidence in the literature (3, 15, 16) based on alpha amino nitrogen determinations and

[1] Glutamine, asparagine, and tryptophan were not determined in these plasmas, so that the totals given in the table are probably about 8 mg. per cent too low.

microbiological assay that neither the total quantity nor the distribution of amino acids in normal urine can be correlated closely with the dietary intake of protein. This fact has been further established in the present investigations by two types of experiments. In the first, a normal individual was maintained for one week intervals on diets containing 15 Gm., 70 Gm., and 200 Gm. of protein, and aliquots of 24-hour urine samples taken during the middle of the experimental period were chromatographed (Table IV). A 13-fold increase in dietary protein causes, for the most part, a 2 or a 3-fold increase in the excretion of any individual amino acid (1-methylhistidine excepted). Moreover, the total quantity of amino acids excreted also only doubles, if the contribution of 1-methylhistidine is neglected. 1-Methylhistidine is the only amino acid the excretion of which in these experiments reflects in a roughly linear fashion the level of protein in the diet.

Evidence for a lack of dependence of the level of urinary amino acids upon the amount of protein ingested is afforded by analyses of normal fasting and normal post-prandial urine. In two instances, both the qualitative and quantitative distribution of amino acids in normal fasting urine were found, on an hourly basis, to be similar to, not lower than the 24-hour values already published (5). Moreover, a sample of urine collected three hours after ingesting 50 Gm. of casein (as Protinal)[2] still gave the usual amino acid distribution and quantitative values not higher than some of the normals previously investigated.

In contrast to the normal, however, both the qualitative distribution and the quantitative level of the urinary amino acids in Wilson's disease were found to be markedly dependent upon the dietary intake of protein, a finding somewhat at variance with a conclusion of Cooper, Eckhardt, Faloon, and Davidson (3). The data presented in Figures 4 and 5 of the preceding paper (7), and in Table V of this paper show the levels of amino acids in the fasting urine from two patients with Wilson's disease, and in a urine specimen collected from one of these patients after the ingestion of 50 Gm. of casein. The amino acid distribution in fasting urine

TABLE V

Effect of a casein meal upon the amino acid levels of the urine in Wilson's disease

Amino acid	Urine levels calculated as mg. amino acid excreted per 24 hrs.		
	Fasting Patient 9	Fasting* Patient 7	Post protein† Patient 7
Asparagine	204	104	314
Glutamic acid	30	6	31
Aminoadipic acid	7	12	45
Glycine + citrulline‡	596	328	586
Alanine	190	60	434
Aminobutyric acid	<10	<10	12
Valine	14	6	230
Leucine	28	20	152
Isoleucine	14	12	53
Serine	338	173	550
Threonine	379	141	600
Half-cystine	124	41	117
Methionine	<10	<10	29
Taurine	34	8	20
Proline	<10	<10	197
Phenylalanine	38	19	110
Tyrosine	144	48	480
Histidine	500	286	516
1-methylhistidine	<10	38	55
3-methylhistidine	42	40	33
Ornithine	13	<10	<10
Lysine	116	53	395
Arginine	8	5	165

* Urine volume, 350 cc. in 4 hours.
† Urine volume, 780 cc. in 3 hours.
‡ Calculated as glycine.

TABLE VI

The effect of a casein meal upon the amino acid levels in the plasma of a normal individual and a patient with Wilson's disease

Amino acid	Plasma levels in mg. of amino acid per 100 cc.			
	Post protein		Increase after protein	
	Normal	Patient 7	Normal	Patient 7
Asparagine	0.89	—	0.31	—
Glutamic acid	1.80	1.48	0.82	0.83
Aminoadipic acid	<.10	<.10	0	0
Glycine + citrulline*	1.57	1.92	0.09	0.37
Alanine	4.64	4.42	1.08	2.22
Aminobutyric acid	0.24	0.39	0.09	0.17
Valine	6.38	8.45	3.44	5.94
Leucine	5.08	6.00	3.34	4.52
Isoleucine	3.03	3.44	2.02	2.71
Serine	1.80	1.63	0.83	0.76
Threonine	2.26	2.39	0.97	1.28
Half-cystine	1.49	1.59	−0.02	0.20
Methionine	0.79	1.26	0.49	0.96
Taurine	0.43	0.28	−0.04	0.02
Proline	5.73	6.00	3.53	4.36
Phenylalanine	1.55	2.55	0.83	1.57
Tyrosine	3.03	4.48	1.90	3.55
Histidine	1.60	1.33	0.27	0.42
1-methylhistidine	0.32	<.10	0.06	0
3-methylhistidine	<.10	<.10	0	0
Ornithine	1.22	1.50	0.29	0.72
Lysine	5.50	7.22	2.57	4.52
Arginine	2.30	3.12	0.90	2.05

* Calculated as glycine (cf. footnote to Table III).

[2] A micropulverized casein-carbohydrate mixture, The National Drug Company, Philadelphia.

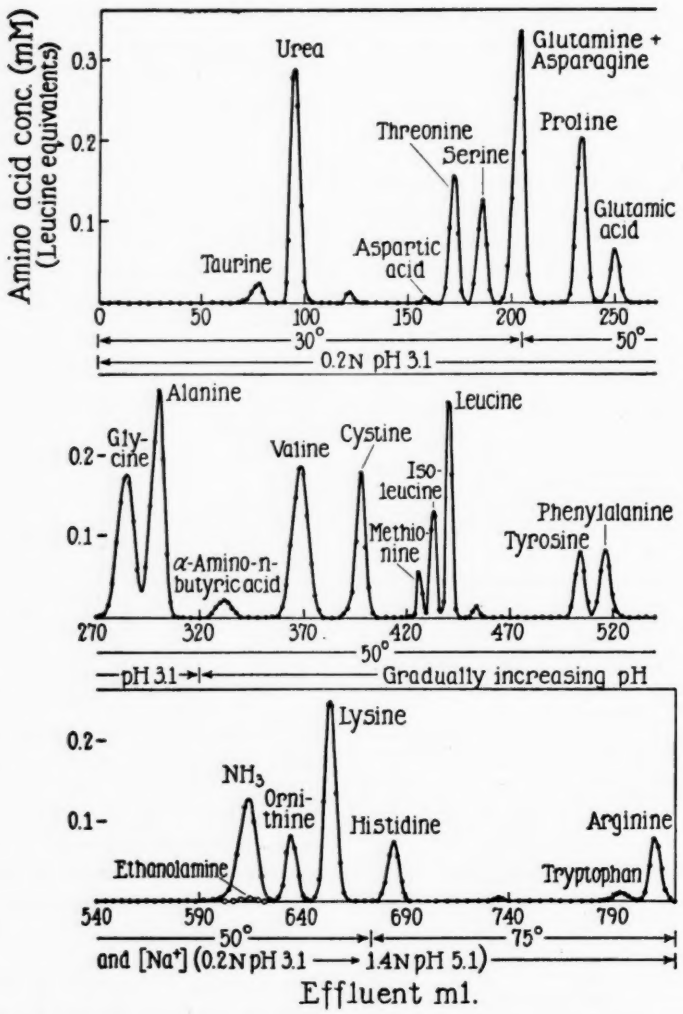

FIG. 2. CHROMATOGRAPHIC SEPARATION OF AMINO ACIDS FROM THE PLASMA OF A PATIENT WITH WILSON'S DISEASE, PATIENT 7, TABLE III

The column of Dowex 50 (4 per cent cross-linking), 0.9 × 150 cm., was operated in the sodium form with buffers of the pH and temperature indicated as eluants. A sample of 8.3 cc. of deproteinized plasma was chromatographed. The open circles under the ammonia peak indicate the quantity of ninhydrin-positive material (ethanolamine) remaining in these fractions after they had been rendered alkaline and exposed to reduced pressure to remove ammonia. Citrulline, shown by Archibald (21) to be present in plasma to the extent of about 0.50 mg. per 100 cc., emerges with glycine in these chromatograms.

is quite different from that observed with normal subjects or from that shown by the 24-hour specimens given in Table I. For glutamic acid, aminoadipic acid, aminobutyric acid, valine, leucine, isoleucine, methionine, proline, phenylalanine, ornithine, and arginine, the levels are essentially normal. Increased amounts of threonine, serine, asparagine, glycine, and cystine (possibly also

glutamine) are present, and somewhat increased quantities of alanine, tyrosine, histidine, and lysine. Taurine excretion is notably small.

After the ingestion of 50 Gm. of protein, however, there is a large rise in all of the urinary amino acids with the exception of aminobutyric acid, taurine, ornithine, and the methylhistidines. Alanine, valine, leucine, threonine, cystine, proline, tyrosine, lysine, and arginine increase the most. The pattern of the rise in urinary amino acid excretion can be correlated only partly with the amino acid composition of the casein fed.

In view of the results obtained with urine, the response of the plasma amino acid level in Wilson's disease to the ingestion of protein was determined. Fasting blood was withdrawn from Patient 7 at the same time as the fasting urine sample (Table V) was taken, and another blood sample was taken two hours after the ingestion of 50 Gm. of casein. The post-protein urine sample was collected during a three-hour period beginning at the time of the ingestion of the casein. Blood samples from a normal individual were taken in the same fashion.

The results of the analyses of fasting and postprandial plasma in the normal subject and in the patient are given in Table VI. In both individuals the plasma levels of almost all amino acids rise after ingesting casein. The levels of taurine, the methylhistidines (too low in amount to be detectable in Wilson's disease plasma), and aminobutyric acid remain constant, or nearly so, which is not surprising since they are not constituent amino acids of casein. Ornithine, on the other hand, which is not normally considered to be a component of casein, increases in amount just as do some of the other amino acids. Cystine, however, which comprises only 0.34 per cent of the casein fed, does not alter at all in concentration in the normal subject, but rises slightly in the patient. The increase in the remaining amino acids reflects to some degree, but not in detail, the amino acid composition of the casein fed. Thus the greatest increase is shown by valine, leucine, proline, tyrosine, and lysine, which are also present in fairly large amounts in casein, but a relatively much smaller proportionate increase is observed for glycine, serine, and histidine. The pattern of the rise is similar in both the normal subject and the patient with Wilson's disease. Although the extent of the increase is uniformly greater for all amino acids in the patient with Wilson's disease, the total quantity of amino acids is similar in the two individuals, and the observed variations are not greater than those to be expected among normal individuals. The conclusion at this juncture seems warranted, therefore, that the amino acid picture in the blood in Wilson's disease is close to normal.

DISCUSSION

This communication, together with similar findings reported previously (3, 4) indicate that a renal lesion exists in Wilson's disease. It would appear difficult to explain on any other basis the existence of aminoaciduria in the presence of a normal qualitative and quantitative distribution of amino acids in the plasma, both fasting and postprandial. Although aminoaciduria is a frequent concomitance, this abnormality is absent in one undoubted case of the disease (Patient 2, Table I) out of the six examined in detail. Other cases of Wilson's disease exhibiting a normal urinary output of amino acids have been reported (3). The conclusion seems justifiable that, whatever the cause, the aminoaciduria is a secondary consequence of some other basic disturbance. This basic disturbance may well be connected with copper metabolism (7, 17, 18), since all the patients in the present group (7), including Patient 2, reveal a high urinary and low plasma level of copper. If the disease progresses, however, the kidney may become involved, and an aminoaciduria might then result. It appears likely that the excretion of all of the amino acids is not affected to the same extent nor at the same time, but that the urinary amino acid pattern gradually changes from one which is quantitatively and qualitatively relatively insensitive to dietary protein intake, to one which reflects to a much greater degree the level of protein in the diet.[3] If the foregoing analysis is correct, it would be expected that the urinary amino acid pattern in

[3] It is of interest that the relative amounts of ten of the amino acids excreted by Patients 1, 3, 4 are in the approximate ratio 1 : 1.7 : 1. For example, the excretion of glycine is 520 mg. for Patient 1, 960 mg. for Patient 3, and 530 mg. for Patient 4, a ratio of 1 : 1.84 : 1.02. This constancy of ratio for so many amino acids might reflect the fact that all three patients were on the same ward and received the same diet. The urine specimens for two of the patients (1 and 3) were collected on the same day, for the third patient, one week later.

a group of individuals suffering from Wilson's disease might vary markedly depending upon the state of the disease and the composition of the diet. Evidence for such variation has been presented in this paper.

Although a renal lesion in Wilson's disease is indicated, the nature of this lesion cannot be delineated with much certainty. Some of the findings are obviously compatible with a "lowered kidney threshold" for amino acids, an explanation that has already been advanced (4). Other features are not so readily explained on this basis. For example, judging from the urinary picture, the amino acids most seriously implicated in Wilson's disease are threonine and cystine. Cystine frequently appears in abnormal quantities in the urine, as has been pointed out by Dent and Harris (2), and is found associated with many different amino acids. In cystinuria the accompanying amino acids are lysine, ornithine, and arginine (12, 13). In Wilson's disease, on the other hand, despite an increased excretion of cystine, lysine, and sometimes ornithine, the arginine level is frequently normal. If, as has been suggested by Dent and Rose (12), these four amino acids are reabsorbed in the tubule by a common mechanism, which is deficient in cystinuria, then these four amino acids should appear together whenever this hypothetical block is present. In one instance, Patient 5 (Table I), cystine, arginine, lysine, and ornithine are all present in grossly abnormal quantities, but in the other cases, the parallelism with cystinuria does not hold.

Cystine is an amino acid which seems to appear in abnormal quantities in the urine in many different conditions (19), and associated with many different amino acids. It may be that a high urinary level of cystine does not indicate failure of tubular reabsorption, but reflects a rather more general kidney dysfunction. If cysteine and glutathione SH groups are essential to normal cellular function, the appearance of cystine in the urine may merely indicate a disturbance in some oxidation-reduction mechanism in the kidney.

It would appear that a failure of tubular reabsorption could not account for the lower than normal excretion observed for several amino acids in many of the patients. The level of taurine is frequently lower than normal, as it is also in cystinuria (13), and the methylhistidines also seem to be excreted in less than normal quantities.

SUMMARY

1. The amino acid distribution in 24-hour specimens of the urine of patients with Wilson's disease has been investigated by chromatography on columns of the ion exchange resin Dowex 50. Of six cases examined, five exhibited a widespread and massive aminoaciduria. One undoubted case of the disease showed an almost normal excretion of amino acids. In general it appears that the highest levels of excretion relative to the normal are reached by threonine and cystine, which may be elevated twenty-fold. The levels of serine, glycine, asparagine (and probably also glutamine), valine, tyrosine, and lysine may rise 5 to 10 fold, while histidine, ornithine, and phenylalanine occur at 2 to 4 times the normal level. Two amino acids not encountered in normal urine, namely proline and citrulline, appear in considerable quantities in many cases. The amounts of aminoadipic acid, methionine, isoleucine, leucine, and arginine seem to be the least abnormal, while on the average there appears to be a diminished excretion of taurine, l-methylhistidine, and 3-methylhistidine.

2. Amino acids in conjugated linkages exist in Wilson's disease urine in quantities about double those obtained from normal urine. There is thus a smaller proportional elevation in the amount of conjugated amino acids than found for the free amino acids.

3. As determined by chromatography on Dowex 50, the amino acid distribution in the plasma of subjects with Wilson's disease is qualitatively and quantitatively normal, both in the fasting state and after a protein meal.

4. Quantitatively and qualitatively, the amino acid levels in the urine of normal individuals are relatively insensitive to the amount of protein in the diet. Moreover, fasting urine in the normal has about the same amino acid composition found for the 24-hour specimen. In Wilson's disease, on the other hand, the level of urinary amino acids is quite sensitive to the protein intake, and fasting urine has a very different amino acid composition from that found for the 24-hour specimen.

5. The available evidence indicates clearly that the aminoaciduria in Wilson's disease is a result

of a renal lesion. This symptom appears, however, to be a secondary consequence of some other basic disturbance that may well be connected with copper metabolism. Hence the urinary amino acid pattern in a group of individuals suffering from Wilson's disease might vary markedly depending both upon the state of the disease and the composition of the diet.

ACKNOWLEDGMENT

The authors would like to acknowledge with gratitude the expert technical assistance of Mrs. Gertrude C. Carey.

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STUDIES CONCERNING THE ROLE OF THE ADRENAL CORTEX IN THE PATHOLOGIC PHYSIOLOGY OF DIABETIC ACIDOSIS

I. TEMPORAL RELATIONS BETWEEN THE METABOLIC EVENTS OF EXPERIMENTAL DIABETIC ACIDOSIS AND THE LEVEL OF ADRENAL CORTICAL FUNCTION [1]

By J. W. McARTHUR, G. A. SMART,[2] E. A. MacLACHLAN, M. L. TERRY, D. HARTING, E. GAUTIER, A. GODLEY, K. A. SWALLOW, F. A. SIMEONE, A. ZYGMUNTOWICZ, E. CHRISTO, J. CREPEAUX, W. W. POINT, AND J. A. BENSON, JR.

(From the Children's Medical Service and the Surgical Service of the Massachusetts General Hospital and the Departments of Pediatrics and Surgery, Harvard Medical School, Boston, Mass.)

(Submitted for publication August 3, 1953; accepted November 25, 1953)

An increase in adrenal activity, often of considerable magnitude, has been found to accompany a large number of clinical disorders including such varied entities as meningococcal meningitis, perforated peptic ulcer and myocardial infarction. Adrenal activation under these circumstances is thought to be an important constituent of a non-specific pattern of response to trauma, the so-called "general adaptation syndrome" of Selye (1), whose acute phase has been termed the "alarm reaction." Among other components of the alarm reaction are a negative nitrogen balance, a negative potassium balance out of proportion to the negativity of the nitrogen balance, an impairment of carbohydrate metabolism with a decreased sensitivity to insulin, and an increased mobilization of fat from the periphery to the liver with a tendency to fasting ketosis (2). It is believed that an important function of this complex of events is the conservation of carbohydrate stores and the utilization of body fat and protein as sources of energy.

While this response is presumably a homeostatic mechanism, possessing survival value for the normal organism, it seems unlikely that the reaction is of equal physiological appropriateness under all circumstances. Conceivably, in an organism with pre-existing disease of tissues participating in the reaction, activation of this response pattern might even be detrimental. The outcome of a given stress situation might then be expected to be a biological resultant between the deleterious action of the specific stress and the various effects of the non-specific response to trauma, certain of which would tend to support the organism and others to operate to its disadvantage.

Inasmuch as the adrenal cortical hormones apparently perform an important function in supporting certain components of the general bodily reaction to damage, an increase in adrenal activity might be expected to accompany those metabolic events dependent upon the cortical hormones for their initiation or maintenance during stress. Therefore, the inclusion of serial estimates of the level of adrenal cortical function in a detailed metabolic description of an organism's response to a specific stress might be expected to assist in the identification of the adrenal-conditioned components of that response. Metabolic phenomena found to occur concomitantly with an increase in the level of adrenal cortical activity could then be subjected to closer scrutiny in order to determine whether they were, in fact, dependent upon increased adrenal activity for their initiation or maintenance. Separation of the beneficial from the harmful effects of the alarm reaction would thus be facilitated.

Diabetic acidosis appeared to be a state whose pathologic physiology might be illuminated by such an investigation, inasmuch as the severity of diabetes is known to be increased, and coma occasionally precipitated, by a variety of types of trauma. Experimental diabetic acidosis was the form of

[1] This investigation was supported (in part) by research grants from the Commonwealth Fund of New York, from the National Institutes of Health, Public Health Service; from the Committee on Research in Endocrinology, National Research Council; and from the American Cancer Society.

[2] Commonwealth Fund Fellow, 1948–49.

stress chosen for these studies the first of which, described in this paper, is a description of the metabolic concomitants of increased adrenal activity during the evolution of experimental ketoacidosis. This has been obtained by correlating estimations of blood and urine chemical constituents, balance measurements and changes in insulin sensitivity with blood counts and urinary corticosteroid excretion rates in depancreatized dogs rendered ketotic by insulin deprivation.

EXPERIMENTAL PROCEDURE

Three parasite-free mongrel bitches selected for their gentleness and adaptability were employed as subjects. Females were used in preference to males because of the greater ease of catheterization, which was further facilitated by dorsal slitting of the perineum. Diabetes was produced by total pancreatectomy and several months were allowed to elapse post-operatively in order to permit subsidence of the adrenal reaction to the surgical trauma. During the recovery period the dogs were gradually accustomed to all of the various procedures to be employed during the performance of the metabolic experiments in order to minimize the stressful effects of the procedures themselves. Care was taken to perform the metabolic experiments only during dioestrus.

Prior to the performance of the metabolic experiments the animals were stabilized on a constant dietary regime together with such insulin as permitted a moderate glycosuria and insured the avoidance of hypoglycemia. The diet employed for the first animal studied (Brownie) consisted of horse meat, raw beef pancreas, bread crumbs, and Brewer's yeast. A number of inadequacies became apparent in this regime: (1) Steatorrhea could not be controlled, even by the administration of raw pancreas in amounts of 200 Gm. per day; (2) The calcium balance was consistently negative; and (3) The liver of the animal became enlarged and at autopsy was found to be infiltrated with fat. Subsequent diets were composed of horse meat and bread crumbs plus Brewer's yeast, choline, cod liver oil, sodium chloride, and calcium lactate. Desiccated pancreas substance (Viokase) was substituted for raw pancreas with abolition of the steatorrhea. Hepatic enlargement no longer occurred, and the absence of fatty degeneration in the liver was established in one animal (Blackie) who came to autopsy.

The whole of the food required for the control and experimental periods was intimately mixed before the study was begun. It was then divided into accurately weighed portions each of which was placed in a separate jar and kept frozen until just before use. The nitrogen and mineral contents of these diets were determined separately for each experiment; these values are summarized in Table III–A, B, C.

Crystalline insulin and food were given every twelve hours to all of the animals except one (Brownie, who was given protamine zinc insulin, crystalline insulin, and food every twelve hours). In the experiments designed to obtain a description of the changes in blood and urine chemical constituents and in metabolic balances, the withdrawal of insulin was preceded by a number of control periods, each of which was three days in length (except in Brownie, whose control periods were twelve hours in length). Food was withdrawn simultaneously from one of the animals (Brownie), but was offered at twelve hour intervals to the other two dogs. The three animals had access to water at all times. Because of the rapidity with which acidosis developed, the length of the subsequent experimental periods was shortened to twelve hours.

The dogs were confined in metabolism cages and all urine, feces, vomitus and uneaten food collected. When, during its decline into acidosis the animal became nauseated, it was lightly harnessed in the cage and a bipartite collecting pan inserted beneath the grated cage floor so that urine and vomitus could be accumulated separately. Jugular blood samples were taken under oil at the beginning of each period before food was offered. The urine collected was stored at 5° C.; all except that destined for corticosteroid analysis was preserved under toluene. The animals were weighed at the beginning of each period on scales with an accuracy of ± 10 grams.

The experiments designed to obtain a description of the changes in insulin sensitivity were, of necessity, conducted separately since it was found that performance of serial sensitivity tests in an animal deprived of its maintenance insulin postponed the onset of ketoacidosis almost indefinitely. Determinations of insulin sensitivity were therefore made in successive experiments upon the same animal (Frisky) after 12, 24, 36, 48, and 60 hours of total insulin deprivation. Upon the completion of each experiment, time was allowed for the dog to regain metabolic equilibrium and insulin sensitivity, if it had been impaired, before its maintenance insulin was again withdrawn.

The insulin sensitivity tests were performed by administering intravenously 10 units of a special preparation of Lilly insulin[3] which was free from the hyperglycemic factor. This comparatively large dose (0.6 units per kilogram) was selected in order that an unequivocal fall in blood sugar might be obtained after prolonged insulin deprivation, when the occurrence of insulin resistance was anticipated. To minimize errors in dosage the insulin was diluted with physiological saline so that one cubic centimeter contained one unit. Blood was drawn for determination of its sugar content just prior to the injection of the insulin and 20, 60, and 90 minutes, thereafter.

It was decided to terminate the observations at 90 minutes because of the demonstration (3, 4) that for a period varying from 30 to 90 minutes after the injection of insulin the blood sugar falls at a virtually uniform rate which bears no close relation to the nutritional condition of the subject. After this period the blood sugar rises much more rapidly in glycogen-rich than in glycogen-depleted subjects, the retarded rate of recovery in the latter

[3] Special insulin No. 2756, for generous supplies of which the authors are indebted to Dr. W. R. Kirtley of the Lilly Research Laboratories.

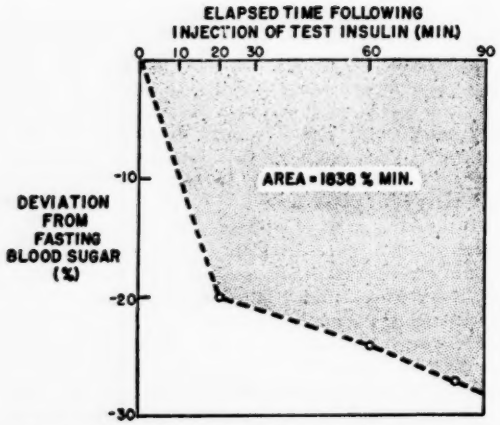

FIG. 1. THE METHOD EMPLOYED FOR COMPUTING THE INDEX OF INSULIN SENSITIVITY, UTILIZING THE RESULTS OF THE SENSITIVITY TEST PERFORMED AFTER 60 HOURS OF INSULIN DEPRIVATION FOR ILLUSTRATION

Elapsed time following injection of test insulin	Blood sugar	Deviation from fasting blood sugar
(*min.*)	(*mg.* %)	(%)
0	570	—
20	458	−20
60	434	−24
90	408	−28

presumably reflecting the occurrence of gluconeogenesis. While it thus appeared that hepatic glycogen depletion would tend to minimize rather than to exaggerate any tendency toward insulin resistance, it was hoped to reduce the influence of this variable by employing a comparatively brief period of observation.

For estimation of the insulin sensitivity upon any given occasion the percentage fall from the fasting level was ascertained for each blood sugar value, this percentage plotted on graph paper, and the area enclosed by the resulting curve determined planimetrically. This area, expressed numerically in "per cent minutes," constitutes an index of sensitivity to the injected insulin (see Figure 1). The area varies directly with insulin sensitivity, and were there an entirely adequate means of correcting for variation in the level of the fasting blood sugar there might be justification for regarding the area as a measure rather than as an index of sensitivity. In computing the area enclosed by the curve, the role of the fasting blood sugar level as a variable has been reduced, but not negated, by expressing subsequent blood sugar values in terms of percentage fall from the fasting level for the given test.

METHODS

Sodium was determined by the method of Butler and Tuthill (5), chloride by a modification of the method of Wilson and Ball (6), potassium by the method of Fiske and Litarczek (7), calcium by the method of Fiske and Logan (8), phosphorus by the method of Fiske and Subbarrow (9), magnesium by a modification of the method of Briggs (10), total nitrogen by the macro-Kjeldahl method of Peters and Van Slyke (11) and glucose and non-protein nitrogen by the methods of Folin (12, 13). Total protein of the serum was estimated by the method of Lowry and Hunter (14), CO_2 content by the method of Van Slyke and Neill (15) and pH by the method of Hastings and Sendroy (16). Measurements of serum fat content were made during the insulin sensitivity studies, the indices employed being the lipokrit (17) which provides a measure of changes in the total lipoid content of the serum, and the chylomicron index (18) which presumably reflects changes in serum neutral fat.

Creatinine was determined by a modification of the method of Bonsnes and Taussky (19). A correction factor has been applied to all of the serum creatinine values reported in Parts I and IIb so as to render them comparable to the serum creatinine values obtained in Part IIa by the use of Hare's (20) method. Hare's method appears to be more specific for creatinine than that of Bonsnes and Taussky, in that it eliminates substances other than creatinine which may give a color with picrate.

Total ketones were measured by a modification of the method of Nanavutty (21) in the experiment on Brownie. In the other experiments acetoacetic acid and acetone were separated from beta hydroxybutyric acid and both fractions subsequently assayed by the salicylaldehyde method of Behre (22). Acetone and acetoacetic acid were removed from a Somogyi $ZnSO_4$–$Ba(OH)_2$ filtrate by acidification and distillation into 2 per cent $NaHSO_3$ for 15 minutes at low heat. Beta hydroxybutyric acid, which still remained, was then oxidized to acetone by refluxing for 15 minutes with acid dichromate and the acetone collected by distillation into a fresh solution of 2 per cent $NaHSO_3$ for 20 minutes. Recoveries from standard solutions of these constituents were 70 per cent for acetone and acetoacetic acid and 50 per cent for beta hydroxybutyric acid; recoveries from standards added to a filtrate of normal human blood were 66 per cent for acetone and acetoacetic acid and 52 per cent for beta hydroxybutyric acid. All values reported have been corrected to compensate for these losses, which have been assumed to be 30 per cent for acetone plus acetoacetic acid and 50 per cent for beta hydroxybutyric acid. The acetone derived from the acetoacetic acid-acetone fraction has been expressed as millimols of acetoacetic acid; that from the beta hydroxybutyric acid fraction as millimols of beta hydroxybutyric acid.

Hematologic changes and alterations in urinary corticosteroid excretion were employed as indices of the level of adrenal cortical function. Circulating blood eosinophils and total white cells were counted in the double-celled Fuchs-Rosenthal chamber by a modification of the method of Randolph (23). A solution of 0.025 per cent phloxine B in propylene glycol was employed as the diluent and all eosinophils in both cells of two chambers

enumerated. Lymphocyte and neutrophil counts were made from a Wright-stained blood smear, a total of 500 cells being counted. The urinary corticosteroids were measured by the method of Talbot, Saltzman, Wixom, and Wolfe (24).

RESULTS

I. *General Metabolic Experiments*

(a) *Clinical course*

The clinical course of all three animals following the discontinuation of insulin therapy was very similar. Hedon's serial photographs (25) of his dog as he lapsed into acidosis after insulin withdrawal are an admirable representation of the changes in physical appearance. Our animals differed from Hedon's only in their more rapid decline during insulin deprivation. Polydipsia and polyuria were noted to begin within a few hours after insulin withdrawal, anorexia and muscular weakness in 48 hours, vomiting in 60 hours, somnolence and Kussmaul respiration in 72 hours. If treatment were delayed until 72 hours had elapsed following the omission of insulin, death ensued in spite of vigorous efforts to save the animals with insulin and intravenous fluids. One animal (Frisky), in whom therapy was begun after 60 hours of insulin deprivation, survived. Despite their serious condition in the later stages of ketosis, the dogs did not become comatose until just before death, a fact which obscured their urgent need for treatment. An incidental observation of some interest was the occurrence of hemolytic crises [4] in two of the dogs during last stages of ketoacidosis.

(b) *Indices of adrenal cortical function*

The hematologic data and urinary corticosteroid excretion values are summarized in Table I and are presented in graphic form for one animal in Figure 2. Changes in these indices immediately after insulin withdrawal were slight. However, as insulin deprivation continued, the eosinophil count tended to rise somewhat.

Terminally, when the animals were prostrate, vomiting, and rapidly approaching death, all the indices pointed to an increase in the level of adrenal cortical function. Both eosinophil and lymphocyte counts fell, and the neutrophil count rose abruptly. The resultant of these changes was a pronounced increase in the total white cell count. It seems probable that the eosinopenia and lymphopenia and the leukemoid reaction (26, 27) sometimes reported in diabetic coma patients not suffering from infection are analogous phenomena, ascribable to the alarm reaction. The rate of corticosteroid excretion underwent a terminal increase in two of the three animals and declined just prior

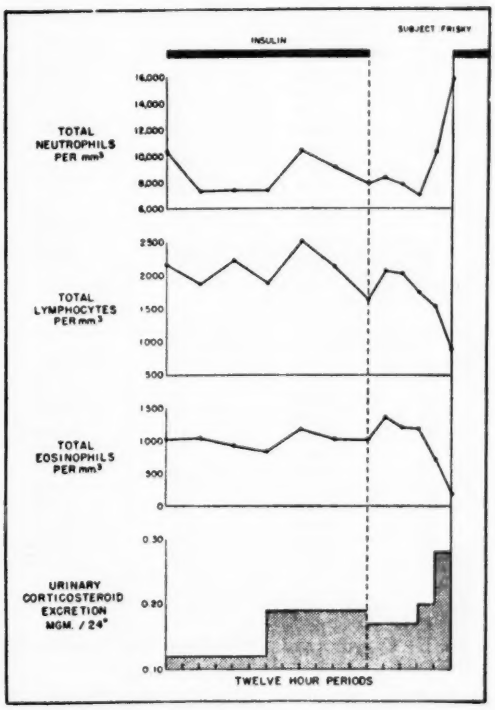

FIG. 2. CHANGES IN THE NUMBER OF CIRCULATING NEUTROPHILS, LYMPHOCYTES AND EOSINOPHILS AND IN THE RATE OF URINARY CORTICOSTEROID EXCRETION BEFORE AND AFTER WITHDRAWAL OF INSULIN FROM A DEPANCREATIZED DOG

[4] In one animal (Brownie) hemoglobinemia and hemoglobinuria were observed before treatment had been started; in the other animal (Frisky), they appeared shortly after the institution of insulin and saline therapy and continued for 24 hours. Methemalbumin was demonstrated spectroscopically in the serum of both animals, indicating that the site of hemolysis was, in all probability, intravascular. Similar hemolytic crises were reported by Hedon to have occurred in his dog during two acidotic episodes; they have also been observed by Long (28) in diabetic cats permitted to become ketotic. Although frank hemolysis has never, to our knowledge, been observed in human beings suffering from diabetic acidosis, Guest (29) has found the osmotic fragility of the erythrocytes of such patients to be increased.

TABLE I

Changes in hematologic and chemical indices of adrenal cortical function before and after the withdrawal of insulin from depancreatized dogs

Date	Time	Period*	Blood				Urinary corticosteroids	Clinical notes
			Total WBC	Neutrophils	Lymphocytes	Eosinophils		
			per mm.³				*mg./24 hrs.*	
A. Brownie								
Nov. 12	6 am	C I				844		
	6 pm	II				625	0.10	
13	6 am	III				475		
	6 pm	IV				862	0.17	
14	6 am	V				638		
	6 pm	E I				294	0.03	Insulin omitted
15	6 am	II				694		
	6 pm	III				762	0.09	
16	6 am	IV				956		
	6 pm	V				406	0.32	
17	6 am	VI				37		
	6 pm	VII				12		Death
B. Blackie								
Mar. 16	6 am	C I	12,600			981		
17	6 am	I	14,496			1,231	0.14	
18	6 am	I	13,000			950		
19	6 am	C II	12,240			1,075		
20	6 am	II	12,000			1,250	0.13	
21	6 am	II	11,666			1,150		
22	6 am	E I	13,775			1,050	0.20	Insulin omitted
	6 pm	II	11,000			1,350		
23	6 am	III	10,450			1,725	0.33	
	6 pm	IV	10,866			1,659		
24	6 am	V	10,100			1,741		
	9 am	V	—			2,428	0.26	
	2 pm	V	12,250			1,891		
	6 pm	VI	10,800			1,564	0.16	
25	6 am	VII	29,800			388		Death
C. Frisky								
Apr. 18	6 pm	C I	14,560	10,367	2,155	1,033		
19	6 pm	I	11,580	7,341	1,853	1,047	0.12	
20	6 pm	I	11,900	7,449	2,213	928		
21	6 pm	C II	11,080	7,424	1,884	843		
22	6 pm	II	14,880	10,327	2,500	1,175	0.19	
23	6 pm	II	13,500	8,999	2,133	1,027		
24	6 pm	E I	11,700	7,839	1,638	1,012		
25	6 am	II	12,840	8,346	2,054	1,360	0.17	Insulin omitted
25	6 pm	III	11,990	7,805	2,026	1,215		
26	6 am	IV	11,190	7,195	1,746	1,190	0.20	
26	6 pm	V	13,540	10,399	1,516	712	0.28	
27	6 am	VI	18,690	15,887	897	197		Treatment begun

* C—Control periods.
E—Experimental periods (after insulin withdrawal).

to death in one animal (Blackie) after having risen previously. In no case did the corticosteroid excretion rate increase to the extent previously observed (30) in human beings. In the earlier study it was found that the rate of corticosteroid excretion by patients suffering from diabetic acidosis of moderate severity was two to eight times as rapid as that following recovery; in this study the rate during acidosis was, at most, slightly more than twice as rapid as the control rate. At least

two factors appear to be responsible for the discrepancy: (*1*) The great rapidity with which the animals declined into ketoacidosis, denying to the dogs' adrenals sufficient time to achieve maximal activity prior to death; and (*2*) the renal impairment manifested by all the animals in the terminal phases of ketoacidosis. As Marks and Leaf (31) have shown, experimental reduction in the glomerular filtration rate of the dog is associated with a pronounced reduction in renal clearance of corticosteroids.

(c) *General metabolic observations*

(1) *Blood, plasma, and serum constituents.* The changes in the blood, plasma and serum constituents which occurred in the three animals during the evolution of ketoacidosis are summarized in Table II. Additional data concerning changes in plasma CO_2 content, blood ketones and serum lipoids were secured in the experiments concerned with changes in insulin sensitivity and are shown in Table VIII.

The blood changes bear a qualitative, but a not always quantitative, resemblance to those classically established in human subjects. The blood sugar increased to a level between 200 and 300 mg. per cent immediately after insulin withdrawal and remained in this range until the condition of the animal became critical, when again it rose abruptly. The serum CO_2 content and pH declined as vomiting began. However, the depression was not comparable to that observed in human diabetic acidosis of equivalent clinical severity. An increase in the level of circulating lipoids preceded the rise in blood ketones; the ketone levels finally attained were roughly comparable to those found clinically in diabetic coma. It should be noted that there is a considerable difference between the range of ketone values obtained by the Behre and Nanavutty methods, particularly with respect to urinary ketone values. The Behre method yielded results in good agreement with those obtained by investigators (32) using the method of Van Slyke (33) and by Dixon, Comfort, Lichtman, and Benson (34) using the method of Greenberg and Lester (35). However, these values are only about half as high as those obtained by the Nanavutty method. No reason for the discrepancy is apparent; it appears to reflect the unsatisfactory state of ketone methodology.

(2) *Metabolic balances.* The metabolic balance data are presented in detail in Table III and in summary form in Table IV. All of the balances (except that of sodium) were similar to those reported to occur after the withdrawal of insulin from human subjects.

The balances of nitrogen, potassium, phosphorus, and magnesium became negative as soon as insulin was withdrawn. The negativity of the nitrogen, phosphorus, and potassium balances underwent a further increase terminally, in association with evidences of increased adrenal activity. The rate of loss of magnesium, on the other hand, remained much the same throughout the experiment. In Figure 3–A, B, C the balances of nitrogen, potassium, and phosphorus are represented graphically. The ordinate scales of the figures are so correlated that all of the columns coincide when these substances are lost or retained in the proportions in which they exist in protoplasm, 2.78 mEq. of potassium and 63 mg. of phosphorus being

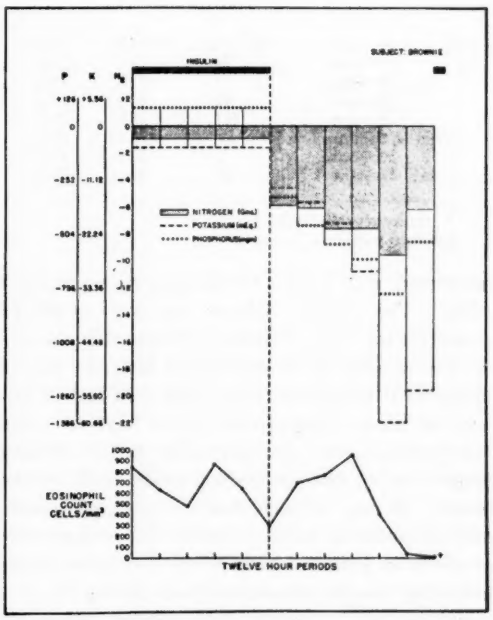

FIG. 3–A. METABOLIC BALANCES OF NITROGEN, POTASSIUM, AND PHOSPHORUS AS RELATED TO THE EOSINOPHIL COUNT BEFORE AND AFTER THE WITHDRAWAL OF INSULIN FROM A DEPANCREATIZED DOG

TABLE II

Blood, plasma and serum values before and after the withdrawal of insulin from depancreatized dogs

Date and time	Beginning of period	Sugar	CO_2 content	pH	Hgb.	Hct.	Total protein	NPN	Creatinine	Total ketones
		mg. %	*mEq./L.*		*Gm. %*	*%*	*Gm. %*	*mg. %*	*mg. %*	*mM/L.*
A. Brownie										
November 12—am	C I	53	27.1	7.40	15.7	48	6.8	72	—	0*
13—am	III	54	26.2	7.38	14.2	48	6.5	59	—	0
14—am	V	217	24.5	7.42	13.3	45	6.7	74	—	0
14—pm	E I	303	26.4	7.44	14.4	45	6.8	45	—	0
15—am	II	286	25.0	7.40	13.3	45	6.9	70	—	0
15—pm	III	274	22.0	7.39	13.7	45	6.7	83	—	4.0
16—am	IV	247	19.7	7.40	13.5	45	6.7	96	—	8.8
16—pm	V	258	16.7	7.35	14.3	47	6.1	98	—	11.6
17—am	VI	320	12.3	7.32	13.3	44	5.9	109	—	18.0
17—pm	VII	388	12.9	7.27	14.6	47	6.7	141	—	24.8
18—am	(Post mort.)	513	—	—	—	—	—	149	—	26.4
B. Blackie										
March 16—am	C I	202	25.4	7.45	16.5	52	6.8	44	0.42	0.6†
19—am	II	238	26.0	7.47	16.3	51	6.7	39	0.48	0.3
22—am	E I	189	25.5	7.47	15.5	49	6.6	38	0.50	0.3
22—pm	II	238	22.7	7.47	15.7	49	6.4	44	0.48	0.3
23—am	III	288	22.2	7.48	14.9	45	6.4	40	0.60	0.6
23—pm	IV	310	24.1	7.40	14.4	43	5.8	44	0.52	4.2
24—am	V	282	21.7	7.48	13.5	41	5.7	34	0.47	3.8
24—pm	VI	263	20.9	7.45	13.7	—	5.7	34	0.49	6.0
25—am	VII	364	11.4	7.40	15.2	51	6.0	56	0.78	7.8
25—am	(Post mort.)	392	5.5	7.18	16.5	46	6.5	94	1.56	52.1
C. Frisky										
April 18—pm	C I	189	23.8	7.43	17.0	51	7.0	42	0.58	0.2†
21—pm	II	137	24.4	7.43	16.5	52	6.7	35	0.63	0.6
24—pm	E I	220	24.1	7.43	16.3	50	6.7	29	0.63	0.5
25—am	II	278	23.2	7.43	16.1	49	5.9	32	0.56	1.9
25—pm	III	288	21.1	7.40	16.3	48	5.7	31	0.55	2.5
26—am	IV	253	21.0	7.41	15.7	44	5.1	29	0.53	2.8
26—pm	V	223	21.8	7.39	14.9	44	4.8	27	0.63	3.1
27—am	VI	231	20.9	7.38	15.5	47	4.7	36	0.69	4.3

* Nanavutty method.
† Behre method.

associated with 1 Gm. of nitrogen in dog muscle (36). The calcium balance has been employed to correct the total phosphorus balance for changes associated with the metabolism of bone, 1.0 mg. of phosphorus being associated with 2.23 mg. of calcium in bone. Magnesium values have not been represented, since an acceptable factor relating magnesium to nitrogen in dog tissue could not be found. It may be seen that nitrogen, potassium, and phosphorus were excreted in protoplasmic proportions except during the period immediately following insulin withdrawal and during the terminal periods. During these periods potassium was excreted in excess of nitrogen.

The initial loss of excess potassium, which occurred in association with a transient intensification of the glycosuria, seems logically attributable to the breakdown of hepatic glycogen. In Brownie, an animal whose liver glycogen stores were presumably reduced as a consequence of dietary inadequacies and steatorrhea, both glucose and potassium were excreted in comparatively small amounts upon the withdrawal of insulin.

The terminal loss of potassium, presumably from the cells of the body as a whole, occurred in association with evidences of adrenal activation. The temporal relation between the two events was an intimate one. Eosinopenia appeared during a different experimental period in each of the dogs and was accompanied in each instance by a loss of excess potassium.

The dogs appeared to differ materially from hu-

TABLE II—*Continued*

Blood, plasma and serum values before and after the withdrawal of insulin from depancreatized dogs

Beta hydroxy-butyric acid	Aceto-acetic acid	Na	Cl	K	Ca	P	Mg	Eosinophil count	Clinical notes
mM/L.		*mEq./L.*	*mEq./L.*	*mEq./L.*	*mg. %*	*mg. %*	*mEq./L.*	*cells/mm.*[3]	
				A. Brownie					
—	—	154	109	4.7	8.6	4.9	1.7	844	
—	—	150	108	4.4	9.1	5.1	1.7	475	
—	—	147	107	4.5	9.1	4.3	1.7	638	
—	—	144	99	4.7	8.8	4.5	1.5	294	Insulin omitted
—	—	145	102	4.7	8.8	4.5	1.6	694	
—	—	145	105	5.0	9.3	4.5	1.3	762	
—	—	147	99	5.3	8.8	6.2	1.1	956	Vomiting begins
—	—	143	97	5.1	8.6	4.5	1.3	406	
—	—	133	86	5.3	7.2	5.7	1.9	37	
—	—	129	79	4.1	5.6	8.7	2.4	12	Treatment instituted
—	—	109	65	26.3	6.8	32.8	4.7	—	Death
				B. Blackie					
0.5	0.1	141	99	5.8	8.8	3.2	1.5	981	
0.2	0.1	141	101	5.6	8.6	3.1	1.4	1075	
0.2	0.1	143	101	5.5	9.1	3.2	1.5	1050	Insulin omitted
0.2	0.1	141	103	5.1	8.6	4.2	1.5	1350	
0.4	0.2	140	100	5.5	8.6	3.9	1.4	1725	
3.4	0.8	141	104	6.0	8.2	4.0	1.0	1659	
2.2	1.6	141	104	4.9	8.1	4.9	0.9	1741	Vomiting begins
3.8	2.2	141	103	4.4	7.2	4.6	0.9	1564	
5.2	2.6	131	88	5.9	4.1	8.3	2.3	388	Treatment instituted
45.5	6.6	126	88	8.2	3.2	16.7	2.9	47	Death
				C. Frisky					
0.2	0	139	105	5.4	8.9	4.5	1.7	1033	
0.3	0.3	145	107	5.1	9.1	4.5	1.7	843	
0.4	0.1	142	105	4.8	9.6	3.4	1.7	1012	Insulin omitted
1.5	0.4	143	105	4.8	9.4	3.8	1.5	1360	
1.6	0.9	138	102	4.5	9.6	4.0	1.5	1215	
1.6	1.2	138	103	5.0	8.0	3.2	0.9	1190	Vomiting begins
1.4	1.7	136	97	3.9	7.8	3.6	2.8	712	
1.8	2.5	134	89	4.2	7.1	4.2	2.0	197	Treatment instituted

man beings in their capacity to conserve sodium. Despite mounting ketosis, two of the three [5] animals were able to effect a marked reduction in urinary sodium and chloride excretion. Herein would appear to lie the explanation of the comparative mildness of canine acidosis. To what extent the dog's capacity to spare base depends upon the rapidity with which it can achieve maximal ammonia production (37) and to what extent it depends upon other factors cannot be stated at present.

Calcium balances became negative late in the experiment in the two animals (Frisky and Blackie) whose calcium intake was sufficiently generous to allow this phenomenon to become apparent.

(3) *Renal clearance studies.* Measurement of blood creatinine levels and of urinary creatinine excretion were made in two of the animals studied. This permitted calculation of the endogenous creatinine clearance, which in the dog can be regarded as a valid index of the glomerular filtration rate. Electrolyte clearances could then be derived and certain inferences regarding tubular, as well as glomerular, function made.

In Tables V–A and B, the renal clearances of creatinine, sodium and potassium are presented together with the eosinophil count at the beginning of each metabolic period. In Frisky, an animal deprived of insulin for only 60 hours, the glomerular

[5] Brownie, who manifested an increasing rate of sodium loss as the rate of ketone excretion increased, had an elevated level of blood non-protein nitrogen even during the control periods which was unexplained; she may have had impairment of the renal ammonia-producing mechanism as well.

TABLE III

Metabolic balance data obtained before and after the withdrawal of insulin from depancreatized dogs

Date	Period	Insulin dosage	Body weight	INTAKE: Food N_2	Na	Cl	K	Ca	Mg	P
		units	*Kg.*	*Gm.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mg.*
November			A. Brownie							
12 am–pm	C I	—	18.77	—	—	—	—	—	—	—
12 pm–13 am	II	P27, R48	—	22.8	80.8	86.5	65.0	9.2	21.6	2200
13 am–13 pm	III	—	18.07	—	—	—	—	—	—	—
13 pm–14 am	IV	P26, R40	—	22.8	80.8	86.5	65.0	9.2	21.6	2200
14 am–14 pm	V	—	18.32	—	—	—	—	—	—	—
14 pm–15 am	E I	—	17.78	—	—	—	—	—	—	—
15 am–15 pm	II	—	17.14	—	—	—	—	—	—	—
15 pm–16 am	III	—	17.19	—	—	—	—	—	—	—
16 am–16 pm	IV	—	16.83	—	—	—	—	—	—	—
16 pm–17 am	V	—	16.76	—	—	—	—	—	—	—
17 am–17 pm	VI	—	16.70	—	—	—	—	—	—	—
17 pm	Treatment begun		16.64							
March			B. Blackie							
16–19	C I	R61	13.12	64.2	421.8	407.4	198.0	178.1	53.8	4737
19–22	II	R61	13.35	64.2	421.8	407.4	198.0	178.1	53.8	4737
22 am–22 pm	E I	—	13.38	10.7	70.3	67.9	33.0	29.7	9.0	789
22 pm–23 am	II	—	13.30	10.7	70.3	67.9	33.0	29.7	9.0	789
23 am–23 pm	III	—	12.95	10.7	70.3	67.9	33.0	29.7	9.0	789
23 pm–24 am	IV	—	13.04	7.7	50.6	48.9	23.7	21.4	6.5	568
24 am–24 pm	V	—	12.84	1.2	7.8	7.5	3.7	3.3	1.0	87
24 pm–25 am	VI	—	12.60	0.4	2.9	2.8	1.4	1.2	0.4	33
25 am	Treatment begun		12.47							
April			C. Frisky							
18–21	C I	R40	17.24	60.8	341.3	392.8	135.4	95.3	41.9	3918
21–24	II	R35	17.24	60.8	341.3	392.8	135.4	95.3	41.9	3918
24 pm–25 am	E I	—	17.25	10.1	56.9	65.5	22.6	15.9	7.0	653
25 am–25 pm	II	—	17.15	10.1	56.9	65.5	22.6	15.9	7.0	653
25 pm–26 am	III	—	17.28	7.8	43.7	50.3	17.3	12.2	5.4	503
26 am–26 pm	IV	—	17.16	0.4	2.2	2.5	0.9	0.6	0.3	25
26 pm–27 am	V	—	16.92	4.9	27.7	31.9	11.0	7.7	3.4	319
27 am	Treatment begun		16.73							

‡ Weight obtained by extrapolation.

TABLE III—*Continued*

Metabolic balance data obtained before and after the withdrawal of insulin from depancreatized dogs

Date	Period	OUTPUT: Urine Vol.	N_2	Na	Cl	K	Ca	Mg	P	Glucose	Creatinine	Total ketones	Beta hydroxybutyric acid	Acetoacetic acid
		cc.	*Gm.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mg.*	*Gm.*	*mg.*		*mM*	
November					A. Brownie									
12 am–pm	C I	155	5.0	26.8	20.8	18.0	0.0	0.1	439	0.8	—	0*	—	—
12 pm–13 am	II	750	18.5	35.0	42.0	61.7	0.2	7.2	1822	22.5	—	0	—	—
13 am–13 pm	III	135	4.4	21.3	19.7	18.1	0.0	2.0	382	1.3	—	0	—	—
13 pm–14 am	IV	1210	17.8	45.2	43.2	66.0	0.0	7.4	1705	60.5	—	0	—	—
14 am–14 pm	V	220	4.9	20.9	33.4	11.0	0.3	0.6	226	7.7	—	0	—	—
14 pm–15 am	E I	195	3.6	2.3	6.7	11.0	0.1	3.0	287	19.5	—	0	—	—
15 am–15 pm	II	195	3.8	3.3	3.5	14.2	0.1	2.2	412	17.6	—	1.7	—	—
15 pm–16 am	III	280	5.9	1.8	4.7	18.4	0.1	3.0	529	19.6	—	15.5	—	—
16 am–16 pm	IV	320	5.9	8.2	4.0	29.9	0.2	2.9	605	19.2	—	43.1	—	—
16 pm–17 am	V	910†	7.5	28.2	31.5	59.6	1.1	2.0	766	31.8	—	79.3	—	—
17 am–17 pm	VI	1450†	4.5	31.9	51.5	54.1	1.1	2.1	527	27.5	—	53.5	—	—
17 pm	Treatment begun													
March					B. Blackie									
16–19	C I	3125	60.0	345.0	380.0	179.2	2.9	4.9	2710	52.5	1295	1.4‡	0.9	0.5
19–22	II	2555	55.9	315.0	356.0	168.8	2.6	4.0	2320	23.6	1266	1.5	1.1	0.4
22 am–22 pm	E I	1160	10.8	80.8	81.4	49.4	0.3	0.2	606	47.4	281	0.5	0.3	0.2
22 pm–23 am	II	1456	13.9	76.7	78.8	47.3	0.4	0.3	661	81.8	327	1.0	0.7	0.3
23 am–23 pm	III	1420	11.9	33.3	49.2	39.9	0.3	1.2	632	86.0	263	3.7	2.4	1.3
23 pm–24 am	IV	885	10.1	29.7	34.9	31.1	0.2	0.8	319	59.8	258	5.7	3.1	2.6
24 am–24 pm	V	300	5.1	9.3	5.2	15.5	0.2	0.3	382	14.5	196	4.8	2.9	1.9
24 pm–25 am	VI	410	5.1	5.1	5.8	29.8	0.5	0.2	426	20.2	207	5.6	3.4	2.2
25 am	Treatment begun													
April					C. Frisky									
18–21	C I	1585	42.7	287.0	306.0	137.0	3.9	14.0	2412	16.6	1476	1.1‡	0.8	0.3
21–24	II	1975	55.6	357.0	380.0	150.5	5.6	18.5	3288	29.2	1984	1.4	1.1	0.3
24 pm–25 am	E I	965	10.4	53.4	58.2	44.3	1.3	2.7	736	58.2	359	0.4	0.2	0.2
25 am–25 pm	II	1410	11.3	56.2	55.0	24.1	1.7	2.8	629	84.6	342	2.7	1.2	1.5
25 pm–26 am	III	810	9.5	5.0	8.9	22.3	1.2	2.8	603	48.6	330	5.1	1.5	3.6
26 am–26 pm	IV	870	7.3	11.7	8.7	27.8	0.9	2.5	540	34.8	283	10.0	4.3	5.7
26 pm–27 am	V	800	8.2	8.6	0.8	43.6	0.9	2.0	660	43.0	340	10.3	3.7	6.6
27 am	Treatment begun													

* Nanavutty method. † Urine mixed with vomitus. ‡ Behre method.

TABLE III—*Continued*

Metabolic balance data obtained before and after the withdrawal of insulin from depancreatized dogs

			OUTPUT													
			Feces								Vomitus					
Date	Period	Dry weight	N_2	Na	Cl	K	Ca	Mg	P	Vol.	N_2	Na	Cl	K	Ca	Mg	P
		Gm.	*Gm.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mg.*	*cc.*	*Gm.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mg*
November						A. Brownie											
12 am–pm	C I	—								—	—	—	—	—	—	—	—
12 pm–13 am	II	—								—	—	—	—	—	—	—	—
13 am–13 pm	III	—								—	—	—	—	—	—	—	—
13 pm–14 am	IV	—	13.5	39.6	3.9	9.2	49.8	29.4	780	—	—	—	—	—	—	—	—
14 am–14 pm	V	—								—	—	—	—	—	—	—	—
14 pm–15 am	E I	—								—	—	—	—	—	—	—	—
15 am–15 pm	II	—								—	—	—	—	—	—	—	—
15 pm–16 am	III	—								—	—	—	—	—	—	—	—
16 am–16 pm	IV	—	6.9	16.3	1.6	3.1	24.2	16.8	320	—	—	—	—	—	—	—	—
16 pm–17 am	V	—								—	—	—	—	—	—	—	—
17 am–17 pm	VI	—								37	0.4	1.6	3.7	0.5	0.9	0.3	9
17 pm	Treatment begun																
March						B. Blackie											
16–19	C I	169.4	13.9	28.9	5.3	6.1	242.0	47.1	2910	—	—	—	—	—	—	—	—
19–22	II									—	—	—	—	—	—	—	—
22 am–22 pm	E I									—	—	—	—	—	—	—	—
22 pm–23 am	II									—	—	—	—	—	—	—	—
23 am–23 pm	III	49.5	5.5	3.1	1.3	2.5	67.8	19.8	870	—	—	—	—	—	—	—	—
23 pm–24 am	IV									—	—	—	—	—	—	—	—
24 am–24 pm	V									tr	0.2	0.7	9.1	1.3	1.2	0.6	45
24 pm–25 am	VI									665	1.0	17.5	32.8	3.3	1.6	4.5	167
25 am	Treatment begun																
April						C. Frisky											
18–21	C I	154.0	13.0	12.7	3.1	2.1	82.8	49.7	1703	—	—	—	—	—	—	—	—
21–24	II									—	—	—	—	—	—	—	—
24 pm–25 am	E I									—	—	—	—	—	—	—	—
25 am–25 pm	II									—	—	—	—	—	—	—	—
25 pm–26 am	III	16.1	4.2	3.5	0.7	1.0	19.0	15.3	440	—	—	—	—	—	—	—	—
26 am–26 pm	IV									450	0.4	10.8	28.3	3.0	1.2	1.1	24
26 pm–27 am	V									1410	3.2	19.3	57.2	11.2	7.1	2.6	130
27 am	Treatment begun																

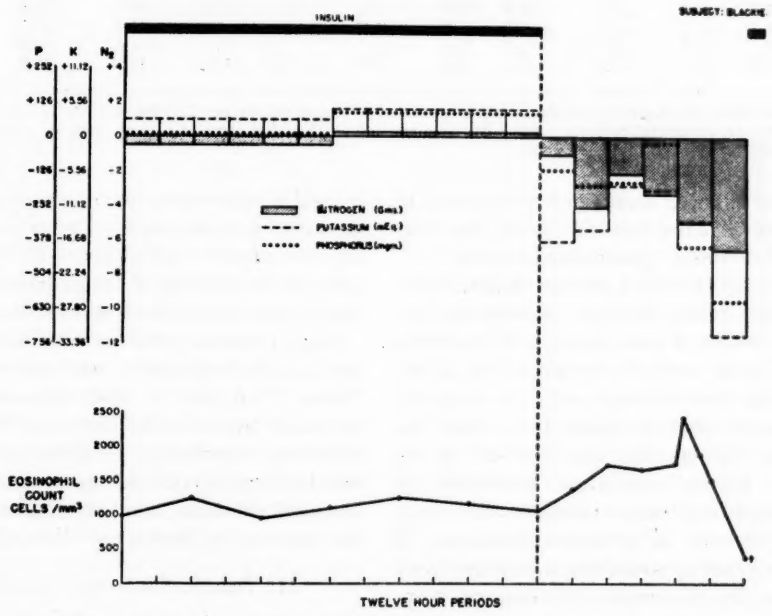

FIG. 3–B. METABOLIC BALANCES OF NITROGEN, POTASSIUM, AND PHOSPHORUS AS RELATED TO THE EOSINOPHIL COUNT BEFORE AND AFTER THE WITHDRAWAL OF INSULIN FROM A DEPANCREATIZED DOG

TABLE IV

*Summary of balance data presented in Table III**

Period	Nitrogen (*Gm.*)			Sodium (*mEq.*)			Chloride (*mEq.*)			Potassium (*mEq.*)		
	Intake	Output	Balance	Intake	Output	Balance	Intake	Output	Balance	Intake	Output	Balance
A. Brownie												
C I	11.4	12.3	−0.9	40.4	36.4	+ 4.0	43.2	32.5	+10.7	32.5	36.5	− 4.0
II	11.4	12.3	−0.9	40.4	36.4	+ 4.0	43.2	32.5	+10.7	32.5	36.5	− 4.0
III	11.4	12.3	−0.9	40.4	36.4	+ 4.0	43.2	32.5	+10.7	32.5	36.5	− 4.0
IV	11.4	12.3	−0.9	40.4	36.4	+ 4.0	43.2	32.5	+10.7	32.5	36.5	− 4.0
V	11.4	12.3	−0.9	40.4	36.4	+ 4.0	43.2	32.5	+10.7	32.5	36.5	− 4.0
E I	0	5.9	−5.9	0	8.9	− 8.9	0	7.4	− 7.4	0	12.5	−12.5
II	0	6.1	−6.1	0	9.9	− 9.9	0	4.2	− 4.2	0	15.7	−15.7
III	0	7.6	−7.6	0	5.9	− 5.9	0	5.1	− 5.1	0	19.2	−19.2
IV	0	7.6	−7.6	0	12.3	−12.3	0	4.4	− 4.4	0	30.7	−30.7
V	0	9.6	−9.6	0	33.9	−33.9	0	35.6	−35.6	0	60.9	−60.9
VI	0	6.2	−6.2	0	36.0	−36.0	0	51.9	−51.9	0	54.9	−54.9
B. Blackie												
C I	10.7	11.2	−0.5	70.3	59.9	+10.4	67.9	63.7	+ 4.2	33.0	30.4	+ 2.6
II	10.7	10.5	+0.2	70.3	54.9	+15.4	67.9	59.7	+ 8.2	33.0	28.6	+ 4.4
E I	10.7	11.8	−1.0	70.3	81.3	−11.0	67.9	81.6	−13.7	33.0	49.8	−16.8
II	10.7	14.8	−4.1	70.3	77.2	− 6.9	67.9	79.0	−11.1	33.0	47.7	−14.7
III	10.7	12.8	−2.1	70.3	33.8	+36.5	67.9	49.4	+18.5	33.0	40.3	− 7.3
IV	7.7	11.0	−3.3	50.6	30.2	+20.4	48.9	35.1	+13.8	23.7	31.5	− 7.8
V	1.2	6.2	−5.0	7.8	10.5	− 2.7	7.5	14.5	− 7.0	3.7	17.2	−13.5
VI	0.4	7.0	−6.6	2.9	23.1	−20.2	2.8	38.8	−36.0	1.4	33.5	−32.1
C. Frisky												
C I	10.1	8.2	+1.9	56.9	48.9	+ 8.0	65.5	51.3	+14.2	22.6	23.0	− 0.4
II	10.1	10.4	−0.3	56.9	60.6	− 3.7	65.5	63.6	+ 1.9	22.6	25.3	− 2.7
E I	10.1	11.2	−1.1	56.9	54.1	+ 2.8	65.5	58.3	+ 7.2	22.6	44.5	−21.9
II	10.1	12.1	−2.0	56.9	56.9	0	65.5	55.1	+10.4	22.6	24.3	− 1.7
III	7.8	10.3	−2.5	43.7	5.7	+38.0	50.3	9.0	+41.3	17.3	22.5	− 5.2
IV	0.4	8.5	−8.1	2.2	23.2	−21.0	2.5	37.1	−34.6	0.9	31.0	−30.1
V	4.9	12.2	−7.3	27.7	28.6	− 0.9	31.9	58.1	−26.2	11.0	55.0	−44.0

* Aggregate data for control periods are here expressed as mean values per 12 hour period for readier comparison with the 12-hour experimental periods. Since Brownie was fed at 24 rather than at 12-hour intervals, her control intake values have arbitrarily been divided by two.

filtration rate was well maintained throughout; in Blackie, deprived of insulin for 72 hours, there was a pre-mortal decline in glomerular filtration.

Both animals exhibited a marked decline in sodium clearance during the later experimental periods. This decline did not appear to be related to an increase in the level of adrenal activity as adjudged by the indices employed. A somewhat closer temporal relation appeared to obtain between changes in potassium clearance and the occurrence of adrenal activation: terminally, as adrenal hyperactivity became manifest, there was a pronounced increase in potassium clearance. If the renal clearance of potassium is expressed as a percentage of the glomerular filtration rate, it appears that at the time eosinopenia began, some influence came into play which resulted in a decreased tubular reabsorption or an increased tubular secretion of potassium. Limitations in present-day investigative techniques (38) unfortunately preclude the drawing of similar inferences with respect to the tubular handling of sodium.

Renal clearance rates for total ketones, acetone and beta hydroxybutyric acid are summarized in Tables VI–A and B. Both animals exhibited a moderate decline in the clearances of all of these substances terminally. Neither acetoacetic nor beta hydroxybutyric acid appeared to behave as a threshold substance, a result in disagreement with that reported by Martin and Wick (39).

II. *Insulin Sensitivity Experiments*

The results of nine tests of insulin sensitivity in control experiments upon the same animal em-

TABLE IV—*Continued*

Summary of balance data presented in Table III

Calcium (*mEq.*)			Magnesium (*mEq.*)			Phosphorus (*mg.*)				
Intake	Output	Balance	Intake	Output	Balance	Intake	Output	Balance	Theoretical P balance based on calcium	P balance minus P based on calcium
					A. Brownie					
4.6	8.4	− 3.8	10.8	8.3	+2.5	1100	1045	+ 55	− 34	+ 89
4.6	8.4	− 3.8	10.8	8.3	+2.5	1100	1045	+ 55	− 34	+ 89
4.6	8.4	− 3.8	10.8	8.3	+2.5	1100	1045	+ 55	− 34	+ 89
4.6	8.4	− 3.8	10.8	8.3	+2.5	1100	1045	+ 55	− 34	+ 89
4.6	8.4	− 3.8	10.8	8.3	+2.5	1100	1045	+ 55	− 34	+ 89
0	8.4	− 8.4	0	7.9	−7.9	0	417	−417	− 75	−342
0	8.4	− 8.4	0	7.1	−7.1	0	542	−542	− 75	−467
0	6.1	− 6.1	0	7.2	−7.2	0	609	−609	− 55	−554
0	6.3	− 6.3	0	7.1	−7.1	0	685	−685	− 57	−628
0	8.1	− 8.1	0	6.5	−6.5	0	855	−855	− 73	−782
0	7.2	− 7.2	0	6.3	−6.3	0	607	−607	− 65	−542
					B. Blackie					
29.7	20.7	+ 9.0	9.0	4.7	+4.3	789	695	+ 94	+ 81	+ 13
29.7	20.6	+ 9.1	9.0	4.6	+4.4	789	630	+159	+ 82	+ 77
29.7	11.6	+18.1	9.0	3.5	+5.5	789	751	+ 38	+162	−124
29.7	11.7	+18.0	9.0	3.6	+5.4	789	806	− 17	+161	−178
29.7	11.6	+18.1	9.0	4.5	+4.5	789	777	+ 12	+162	−150
21.4	11.5	+ 9.9	6.5	4.1	+2.4	568	464	+104	+ 89	− 15
3.3	12.7	− 9.4	1.0	4.2	−3.2	87	572	−485	− 84	−401
1.2	13.4	−12.2	0.4	8.0	−7.6	33	738	−705	−109	−596
					C. Frisky					
15.9	7.6	+ 8.3	7.0	6.4	+0.6	653	544	+109	+ 74	+ 35
15.9	7.8	+ 8.1	7.0	7.2	−0.2	653	690	− 37	+ 73	−110
15.9	5.1	+10.8	7.0	5.8	+1.2	653	824	−171	+ 97	−268
15.9	5.5	+10.4	7.0	5.9	+1.1	653	717	− 64	+ 93	−157
12.2	5.0	+ 7.2	5.4	5.9	−0.5	503	691	−188	+ 65	−253
0.6	5.9	− 5.3	0.3	6.7	−6.4	25	652	−627	− 48	−579
7.7	11.8	− 4.1	3.4	7.7	−4.3	319	878	−559	− 37	−522

ployed for the subsequent insulin deprivation experiments are summarized in Table VII. The tests were performed at intervals over a period of six months. The absolute fall in blood sugar tends to increase with increasing initial values, and the fall expressed as a percentage of the initial value also increases with increasing initial values. These findings accord well with those reported by Radoslav (40), Hemmingsen and Marks (41) and Klatskin (42). Inasmuch as the fasting blood sugars of all but one of the insulin deprivation experiments (that continued for 60 hours) fall within the range of blood sugars obtained in the initial eight control experiments, the mean and standard deviation of the index of insulin sensitivity in these eight have been computed, excluding the ninth experiment.

The results of a series of five experiments on the same animal at the conclusion of progressively longer periods of insulin deprivation are summarized in Table VIII. The observations were not extended beyond deprivation periods of 60 hours since by that time the condition of the animal had become critical and it was feared that further prolongation would result in its death.

No alteration in insulin sensitivity could be detected until deprivation had been continued for 36 hours, but thereafter sensitivity abruptly declined. There was a close temporal relation between the occurrence of eosinopenia and of insulin insensitivity. It seems probable that the sharp rise in blood sugar which occurred between the 48th and 60th hours after insulin withdrawal enhanced insulin sensitivity appreciably. Therefore, the slight further decline in sensitivity which was observed after 60 hours of deprivation in all likelihood represents but a portion of the true decline. Correction for the increased level of blood sugar by ex-

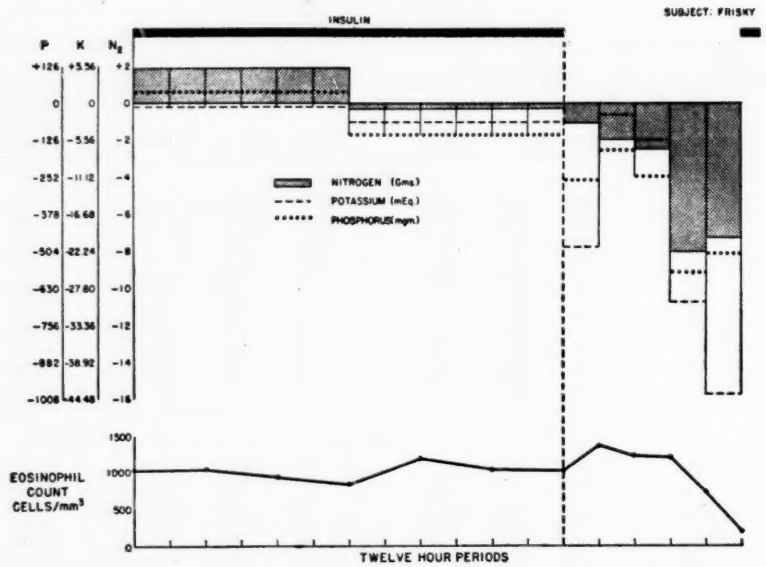

FIG. 3–C. METABOLIC BALANCES OF NITROGEN, POTASSIUM, AND PHOSPHORUS AS RELATED TO THE EOSINOPHIL COUNT BEFORE AND AFTER THE WITHDRAWAL OF INSULIN FROM A DEPANCREATIZED DOG

TABLE V

Renal clearances of creatinine, sodium and potassium as related to the eosinophil count before and after the withdrawal of insulin from depancreatized dogs

Period	Creatinine clearance (GFR)	Sodium clearance	Potassium clearance	$\frac{\text{K clearance} \times 100}{\text{GFR}}$	Eosinophil count at beginning of period
	cc./min.	*cc./min.*	*cc./min.*		*Cells/mm.³*
		A. Blackie			
C I	67	0.56	7	11	981
II	60	0.50	7	12	1075
E I	80	0.77	13	16	1050
II	81	0.74	12	15	1350
III	65	0.32	9	15	1725
IV	72	0.29	8	11	1659
V	57	0.09	5	8	1741
VI	45	0.05	8	17	1564
		B. Frisky			
C I	57	0.46	6	10	1033
II	73	0.56	7	10	843
E I	83	0.51	13	15	1012
II	86	0.54	7	8	1360
III	85	0.50	6	8	1215
IV	68	0.12	9	13	1190
V	72	0.09	15	21	712

TABLE VI

Renal clearances of total ketones, acetoacetic and beta hydroxybutyric acids as related to the creatinine clearance before and after the withdrawal of insulin from depancreatized dogs

Period		Creatinine clearance (GFR)	Total ketone clearance	Acetoacetic acid clearance	Beta hydroxybutyric acid clearance
		cc./min.	*cc./min.*	*cc./min.*	*cc./min.*
		A. Blackie			
C	I	67	0.7	1.6	0.6
	II	60	1.2	1.1	1.3
E	I	80	2.3	2.8	2.1
	II	81	3.5	2.9	3.7
	III	65	2.1	3.4	1.8
	IV	72	2.0	3.0	1.5
	V	57	1.4	1.4	1.3
	VI	45	1.1	1.3	1.0
		B. Frisky			
C	I	57	0.6	0.3	0.9
	II	73	0.6	0.4	0.7
E	I	83	0.4	1.0	0.3
	II	86	1.8	3.4	1.1
	III	85	2.7	4.7	1.3
	IV	68	4.7	5.4	4.0
	V	72	3.9	4.4	3.2

trapolation from the control data is felt to be unwarranted owing to the width of the gap between the highest fasting blood sugar observed during the control studies (246 mg. per cent) and that after 60 hours' insulin deprivation (570 mg. per cent). Despite this difficulty, however, the inference that an additional reduction in insulin sensitivity of uncertain extent occurred terminally seems justified.

DISCUSSION

Of the various components of the metabolic response to the stress of insulin deprivation, a number appear to bear a close temporal relation to the onset of adrenal activation. It is of course recognized that the indices of adrenal cortical activity employed are indirect, and that neither the sensitivity nor the specificity of these indices is fully established. However, within these limitations it would seem that there is a close relation in time between an increase in the level of adrenocortical activity and the following metabolic events: (*1*) An acceleration in the rate of catabolism of protoplasm, manifested by an increased negativity of the nitrogen, potassium, and phosphorus balances; (*2*) a loss of potassium in excess of nitrogen, presumably from the cells of the body as a whole; and (*3*) a decrease in sensitivity to injected insulin. A sudden increase in lipemia and in some, but not all instances, of ketonemia coincided with evidences of increased adrenal activity. However, the levels of both blood lipoids and ketones had begun to rise before eosinopenia became manifest. Any apparent temporal relation between adrenal acti-

TABLE VII

Control observations on the response of the fasting blood sugar of a depancreatized dog (Frisky) to the intravenous injection of 10 units of crystalline insulin*

No. of test	Eosinophil count	Fasting blood sugar	Maximum absolute fall in blood sugar	Maximum per cent fall in blood sugar	Insulin sensitivity 0–90 min.
	no./mm.³	*mg. %*	*mg. %*		*% min.*
1	962	246	168	68	3481
2	819	224	154	64	3369
3	—	206	151	73	3878
4	756	196	123	63	3386
5	837	190	109	54	3089
6	794	179	111	62	2966
7	—	176	98	55	3147
8	900	165	94	53	3165
9	819	103	35	33	1969
Mean of tests 1–8	845	198	—	—	3310
S.D. of tests 1–8	75	27	—	—	287

* 12 hours after the animal's previous feeding and injection of crystalline insulin.

TABLE VIII

The response of the fasting blood sugar of a depancreatized dog (Frisky) to the intravenous injection of 10 units of crystalline insulin together with measurements of the eosinophil count, total leukocyte count, plasma CO_2 content, blood ketone levels, and the serum chylomicron index and lipokrit at the conclusion of progressively longer periods of insulin deprivation

Duration of insulin deprivation (*hrs.*)	0 (Control)	12	24	36	48	60
Fasting blood sugar (*mg. %*)	140	198	229	195	185	570
Insulin sensitivity 0–90 min. (*% min.*)	3,312	3,294	3,294	3,299	1,918	1,838
Eosinophil count:						
Absolute (*no./mm.*^{3})	666	762	769	850	475	25
Change from initial count of individual experiment (%)	—	−4	+19	−1	−34	−92
Total leukocyte count:						
Absolute (*no./mm.*^{3})	9,410	8,500	10,750	10,000	7,800	26,240
Change from initial count of individual experiment (%)	—	−11	+26	−10	−26	+355
Plasma CO_2 content (*mEq./L.*)	22.8	18.0	16.8	17.8	14.0	10.2
Blood ketones:						
Total (*mM/L.*)	0.1	0.1	0.2	1.0	1.2	4.9
Acetoacetic acid (*mM/L.*)	0.0	—	0.1	0.3	0.3	2.4
Beta hydroxybutyric acid (*mM/L.*)	0.0	0.1	0.2	0.8	0.8	2.5
Chylomicron index	23	186	31	414	>1000*	>1000*
Lipokrit (*mg. %*)	1,080	1,104	1,568	1,760	2,680	1,804

* Too dense to photograph for accurate counting.

vation and these metabolic events must, therefore, be regarded as equivocal.

In view of the purely descriptive character of these initial studies, detailed discussion of the results will be reserved to the second of the two papers. A brief digression concerning the possible role of acidosis in these studies is, however, required. As Guest, Mackler, and Knowles (43, 44) have pointed out, acidosis apparently favors, if it does not actually induce, a loss of intracellular constituents such as phosphorus and potassium, and a decrease in sensitivity to injected insulin. Notwithstanding, it seems unlikely that acidosis *per se* figured prominently in the terminal losses of phosphorus and potassium noted in the present experiments. Were these losses due primarily to acidosis, phosphorus as well as potassium might have been expected to have been lost in excess of nitrogen. Rapoport and Guest (45) have, moreover, shown that a pH of less than 7.3 is requisite for the decomposition of phosphoric esters and liberation of inorganic phosphorus from red blood cells. Only one of our animals (Brownie) displayed a pH below this level at any time, and that during the final experimental period.

The mildness of the acidosis which developed during these experiments would also seem to militate against its having exerted a significant effect upon insulin sensitivity. Mackler, Lichtenstein, and Guest (46) have observed that moderate insulin resistance appears in dogs when acidosis is induced by the administration of ammonium chloride. However, the level of the serum CO_2 content at which resistance was observed (6.0 mEq. per liter) is considerably below that attained in the present experiments. Moreover, the possibility that adrenal activation, or other factors, may have contributed to Mackler's results was not excluded.

SUMMARY

1. The temporal relation between adrenal hyperactivity and other metabolic phenomena has been investigated in depancreatized dogs during the evolution of experimental diabetic acidosis by comparison of serial eosinophil counts and urinary corticosteroid determinations with (a) estimations of blood and urine chemical constituents, (b) balance measurements, and (c) changes in insulin sensitivity.

2. Eosinopenia and an increase in the rate of corticosteroid excretion were found to be comparatively late features of ketoacidosis induced by the omission of insulin.

3. A close temporal relation was found to obtain between increased adrenal activity, as judged by these indices, and the following metabolic events: (a) An acceleration in the rate of catabolism of protoplasm, manifested by an increased negativity of the nitrogen, potassium, and phos-

phorus balances; (b) a loss of potassium in excess of nitrogen, presumably from the cells of the body as a whole; and (c) a decrease in sensitivity to injected insulin.

4. An increase in lipemia and, in some instances, of ketonemia was observed to occur in association with evidences of adrenal activation. However, inasmuch as the blood levels of both lipoids and ketones had begun to rise before eosinopenia became manifest, the temporal relation between adrenal activation and these metabolic events must be regarded as equivocal.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to Drs. A. M. Butler and N. B. Talbot for their encouragement and helpful advice; to Drs. T. Hale Ham, Joseph F. Ross and Frank H. Gardner for aid in studying the hemolytic phenomena in the animals; to Drs. Ronald Sniffen and David Freiman for the preparation and study of autopsy material; to Dr. Jane Worcester for advice concerning the mathematical treatment of the data; to Dr. John Kinmonth for help in carrying out the surgical procedures; and to Mrs. Rachel Leech, Mrs. Marguerite Wood, and Mrs. Kirsten Van Loon for assistance in making the chemical determinations.

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STUDIES CONCERNING THE ROLE OF THE ADRENAL CORTEX IN THE PATHOLOGIC PHYSIOLOGY OF DIABETIC ACIDOSIS

II. THE IDENTIFICATION OF ADRENAL-CONDITIONED FACTORS IN THE PHYSIOLOGIC REACTION TO THE STRESS OF INSULIN DEPRIVATION [1]

By J. W. McARTHUR, E. GAUTIER, K. A. SWALLOW, A. GODLEY, E. A. MacLACHLAN, M. L. TERRY, D. HUME, J. CREPEAUX, F. A. SIMEONE, H. KEITEL, AND H. BERMAN

(From the Children's Medical Service and the Surgical Service of the Massachusetts General Hospital and the Departments of Pediatrics and Surgery, Harvard Medical School, Boston, Mass.)

(Submitted for publication August 3, 1953; accepted November 25, 1953)

In a descriptive study designed to correlate changes in the level of adrenal cortical function with other metabolic phenomena occurring during the evolution of experimental diabetic acidosis it was observed (1) that eosinopenia and an increase in the rate of corticosteroid excretion are comparatively late features of ketoacidosis induced by the omission of insulin. A close temporal relation was found to obtain between evidences of increased adrenal activity and the following metabolic events: (a) An acceleration in the rate of catabolism of protoplasm; (b) a loss of potassium in excess of nitrogen; and (c) a decreased sensitivity to injected insulin. In some, but not all, experiments it appeared that adrenal activation was related in time to the occurrence of increased lipemia and ketonemia as well.

In this paper are presented the results of experiments designed to ascertain whether the above events, temporally linked to increased adrenal activity, can, in fact, be regarded as adrenal conditioned. The postulates underlying these experiments are that metabolic reactions dependent upon increased adrenal activity for their initiation or maintenance should be susceptible to: (a) Premature induction by exogenous stimulation of the adrenal cortex during mild stress; and (b) diminution or suppression by maintenance of a fixed supply of adrenal cortical hormones during severe stress.

Accordingly, the experiments comprise a comparison between the metabolic changes which characterize the stressful terminal phases of insulin deprivation in the depancreatized dog and: (a) The changes which accompany the induction of a comparable degree of eosinopenia by the administration of ACTH to a depancreatized dog deprived of insulin for a relatively brief period; and (b) the changes which accompany the withdrawal of insulin from an adrenalectomized-depancreatized animal maintained upon a fixed dose of adrenal cortical hormones.

EXPERIMENTAL PROCEDURE

Response of the depancreatized dog to exogenous stimulation of the adrenal cortex. A balance study was performed upon an animal (Frisky) whose metabolic response to insulin deprivation had been well established by previous withdrawal experiments (1). After being stabilized on the same diet as had been employed for previous balance studies, the animal was deprived of insulin for a period of 36 hours. The eosinophil fall [2] ordinarily occurring from the 48th to the 60th hour after insulin withdrawal was anticipated by administering ACTH intramuscularly in a dose of 20 mg. every three hours between the 24th and 36th hours of deprivation. This particular interval was chosen for adrenal stimulation in order that

[1] This investigation was supported (in part) by research grants from the Commonwealth Fund of New York; from the National Institutes of Health, Public Health Service; from the Committee on Therapeutic Research, Council on Pharmacy and Chemistry, American Medical Association; and from the Ella Sachs Plotz Foundation.

[2] The eosinophil count, rather than the rate of urinary corticosteroid excretion, was employed for determination of the ACTH dosage necessary to simulate the degree of adrenal cortical activity observed in the original insulin deprivation experiments. A reduced clearance of corticosteroids, due to a depression of the glomerular filtration rate during the terminal stages of ketoacidosis, is believed to have resulted in spuriously low levels of corticosteroid excretion.

TABLE I

Blood, plasma and serum constituents preceding and accompanying the administration of ACTH to a depancreatized dog

Date and time	Beginning of period	Clinical notes	Total WBC	Neutrophils	Lymphocytes	Eosinophils	Sugar	CO_2 content	Hgb.
			Cells/mm.³				*mg. %*	*mEq./L.*	*Gm. %*
May 27—9 am	C I		11,560	9,248	1,156	594	340	26.8	16.3
28—9 am			10,400	7,800	1,560	609			
29—9 am	C II		—	—	—	—	270	24.9	16.5
30—9 am			12,460	9,983	1,246	650			
31—9 am	E I	Insulin omitted	11,060	8,516	1,659	594	235	23.6	15.7
9 pm	E II		9,880	7,114	1,482	538	307	22.1	15.6
June 1—9 am	E III	ACTH	10,680	8,117	1,495	562	262	19.5	15.6
12 noon		20 mg.	18,720	14,602	2,059	466			
3 pm		q. 3 h. ×	19,220	15,760	1,922	272			
6 pm		12 h.	23,220	20,666	929	190			
9 pm			20,580	18,728	617	94	275	14.7	15.0

a clear delineation of the effect of exogenous ACTH upon the loss of excess potassium might be obtained. From the results of previous experiments it was known that a loss of excess potassium, presumably associated with hepatic glycogenolysis, would occur during the first 12 hours and that the losses of nitrogen and potassium would be in protoplasmic proportion during the succeeding 24 hours.

Response of the adrenalectomized-depancreatized dog to insulin deprivation. A balance study was performed upon an adrenalectomized-depancreatized dog (Lady) during a 60-hour period of insulin deprivation, a constant supply of adrenal hormones being furnished the animal throughout. The subject was a depancreatized bitch ordinarily requiring 50 to 60 units of crystalline insulin daily for control of the diabetes. Adrenalectomy was performed in two stages, and at autopsy was demonstrated to have been complete. There was no alteration in the severity of the diabetes upon removal of the first adrenal; following the removal of the second adrenal there was little immediate change in the insulin requirement, and desoxycorticosterone acetate was the sole adrenal supplement employed. However, over a period of three weeks the insulin requirement gradually declined to 15 units per 24 hours. Cortisone was then added in amounts sufficient to restore the pre-adrenalectomy insulin requirement. The maintenance regime upon which the animal was ultimately stabilized comprised: (a) 12-hourly feedings containing 30 mEq. of sodium and 65 mEq. each of potassium and chloride, and (b) 12-hourly administration of 25 to 30 units of crystalline insulin subcutaneously, and of 2.25 mg. of desoxycorticosterone acetate and 10 mg. of cortisone acetate intramuscularly. On this regime the dog's weight and serum electrolytes were maintained and the animal exhibited its customary vigor. However, it was vulnerable to all forms of stress. During such episodes there was a marked increase in its sensitivity to insulin, necessitating careful regulation of dosage in order to prevent the occurrence of hypoglycemic reactions. Quantitative tests of insulin sensitivity were not made in this animal inasmuch as the stress of insulin deprivation was accompanied by an increase, rather than a decrease, in sensitivity to injected insulin.

The above maintenance regime was employed throughout the balance study, insulin being withdrawn after two three-day control periods and balance measurements be-

TABLE II

Metabolic balance data preceding and accompanying the administration of ACTH to a depancreatized dog

		INTAKE							OUTPUT						
									Urine						
Date and time	Period	N_2	Na	Cl	K	Ca	Mg	P	Vol.	N_2	Na	Cl	K	Ca	Mg
		Gm.	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mg.*	*cc.*	*Gm.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq*
May 27, 9 am–May 29, 9 am	C I	35.5	273.1	295.4	109.8	82.4	34.0	2584	3095	32.8	251.0	266.7	103.5	8.9	16.4
May 29, 9 am–May 31, 9 am	C II	35.5	273.1	295.4	109.8	82.4	34.0	2584	1987	29.8	243.0	257.7	87.5	5.0	12.0
May 31, 9 am–9 pm	E I	8.9	68.5	73.9	27.4	20.6	8.5	646	1915	9.4	76.6	72.4	59.4	1.7	2.9
May 31, 9 pm–June 1, 9 am	E II	8.9	68.5	73.9	27.4	20.6	8.5	646	1390	11.0	55.5	36.8	32.8	2.4	3.9
June 1, 9 am–9 pm	E III	8.9	68.5	73.9	27.4	20.6	8.5	646	1565	12.4	77.3	20.8	62.6	3.7	4.6

TABLE I—*Continued*

Blood, plasma and serum constituents preceding and accompanying the administration of ACTH to a depancreatized dog

Hct.	NPN	Creatinine	Total ketones	Beta hydroxybutyric acid	Acetoacetic acid	Na	Cl	K	Ca	P	Mg
%	mg. %	mg. %		*mM/L.*		*mEq./L.*	*mEq./L.*	*mEq./L.*	*mg. %*	*mg. %*	*mEq./L.*
54	36	0.61				144	98	4.7	10.3	3.8	1.8
54	43	0.62				142	102	4.7	10.0	3.2	1.7
51	41	0.59	0.8	0.7	0.1	141	104	4.7	9.9	2.8	1.7
50	54	0.70	1.7	1.3	0.4	139	99	3.6	9.8	4.9	1.6
53	52	0.59	3.1	2.0	1.1	142	101	3.9	9.9	4.7	1.5
52.4	53	0.63	4.6	2.3	2.3	141	97	4.0	9.1	4.7	1.8

ing continued throughout five 12-hour experimental periods. It had been hoped that the balance data could be obtained under conditions of fasting, inasmuch as control of food intake presents serious difficulty in anorexic and vomiting animals. However, fasting proved to constitute such a severe auxiliary stress to the depancreatized-adrenalectomized dog that the animal could not withstand insulin deprivation long enough to permit collection of the necessary data. The dog was therefore fed, and the proportion of each feeding retained estimated from the weight of the rejected food and the nitrogen content of any vomitus. By good fortune, the mean percentage of each feeding retained by this dog (59.6) proved almost identical with that retained by a depancreatized animal [3] (62.4), the early vomiting of the adrenalectomized-depancreatized dog being counterbalanced by its lack of anorexia. The corrected food intakes of these two animals during insulin deprivation are presented in Table VI, together with the gross balances of nitrogen, phosphorus, and potassium. Nitrogen balances corrected for changes in blood non-protein nitrogen are also shown; potassium balances have not been similarly corrected since serum charges were negligible.

[3] See metabolic data on Frisky (1).

RESULTS

A. Response of the Depancreatized Dog to Exogenous Stimulation of the Adrenal Cortex

1. *Clinical course.*

The administration of ACTH produced a striking acceleration in the animal's rate of progress toward diabetic acidosis. Polyuria became extremely severe and vomiting began prematurely. When, after a total insulin deprivation period of only 36 hours the experiment was terminated, the dog was moribund.

2. *General metabolic measurements.*

(a) *Blood, plasma and serum constituents.* The changes in the blood, plasma and serum constitu-

TABLE II—*Continued*

Metabolic balance data preceding and accompanying the administration of ACTH to a depancreatized dog

	OUTPUT																		
	Urine						Feces							Vomitus					
P	Glucose	Creatinine	Total ketones	Beta hydroxybutyric acid	Acetoacetic acid	N_2	Na	Cl	K	Ca	Mg	P	N_2	Na	Cl	K	Ca	Mg	P
mg.	*Gm.*	*mg.*		*mM*		*Gm.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mg.*	*Gm.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mg.*
1695	175.6	1117	0.5	0.4	0.1														
618	79.4	1037	0.4	0.3	0.1														
596	119.7	283	2.3	1.4	0.9	11.9	27.0	3.7	4.2	183.0	17.7	1570							
686	83.4	342	17.1	11.7	5.4														
960	66.1	397	53.3	37.4	15.9								3.4	42.7	59.0	10.4	3.7	9.2	217

TABLE III

Summary of balance data presented in Table II

	Nitrogen (*Gm.*)			Sodium (*mEq.*)			Chloride (*mEq.*)			Potassium (*mEq.*)		
Period	Intake	Output	Balance	Intake	Output	Balance	Intake	Output	Balance	Intake	Output	Balance
C I	35.5	37.2	−1.7	273.1	261.0	+12.1	295.4	267.9	+27.5	109.8	105.1	+ 4.7
II	35.5	34.2	+1.3	273.1	253.0	+20.1	295.4	258.9	+36.5	109.8	89.1	+20.7
E I	8.9	10.5	−1.6	68.5	79.1	−10.6	73.9	72.7	+ 1.2	27.4	59.8	−32.4
II	8.9	12.1	−3.2	68.5	58.0	+10.5	73.9	37.1	+36.8	27.4	33.2	− 5.8
III	8.9	16.9	−8.0	68.5	122.5	−54.0	73.9	80.1	− 6.2	27.4	73.4	−46.0

ents which occurred during this experiment are summarized in Table I. Except for a rise in the level of blood ketones and a reciprocal fall in serum CO_2 content which took place during the administration of ACTH these changes were slight.

(b) *Metabolic balances.* The metabolic balance data are presented in detail in Table II and in summary form in Table III. In Figure 1 the balances of nitrogen, potassium, and phosphorus are shown graphically. During the 24 hours of insulin deprivation prior to the administration of ACTH, the balance changes did not differ materially from those observed during comparable phases of previous insulin withdrawal experiments (1). During the 12-hour period of ACTH administration the negativity of the nitrogen, potassium, and phosphorus balances increased abruptly. Nitrogen and phosphorus were lost in roughly pro-

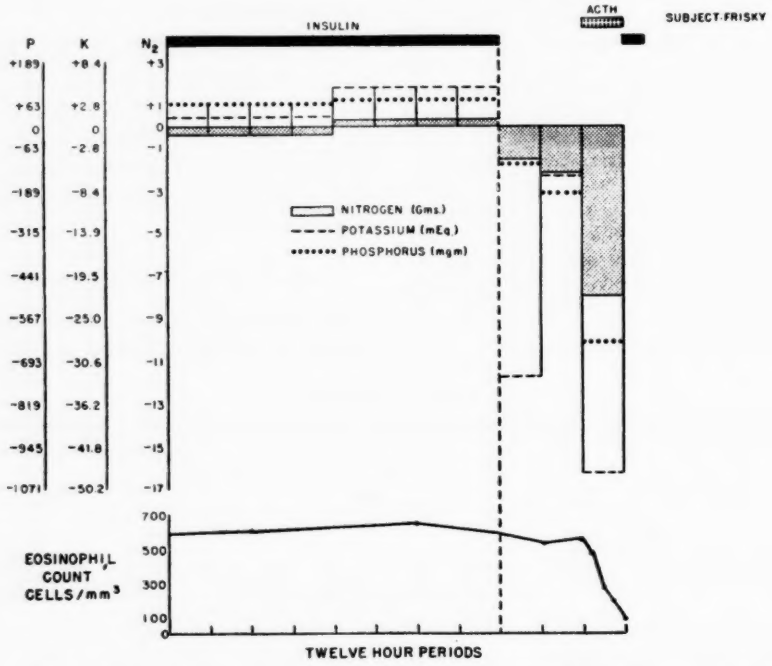

FIG. 1. METABOLIC BALANCES OF NITROGEN, POTASSIUM AND PHOSPHORUS AS RELATED TO THE EOSINOPHIL COUNT BEFORE AND FOR 36 HOURS AFTER THE WITHDRAWAL OF INSULIN FROM A DEPANCREATIZED DOG

During the last 12 hours of insulin deprivation ACTH was administered intramuscularly in a dosage of 20 mg. every three hours.

TABLE III—*Continued*

Summary of balance data presented in Table II

Calcium (*mEq.*)			Magnesium (*mEq.*)			Phosphorus (*mg.*)				
Intake	Output	Balance	Intake	Output	Balance	Intake	Output	Balance	Theoretical balance based on Ca	Protoplasmic balance (Total P minus P based on Ca)
82.4	76.9	+5.5	34.0	22.8	+11.2	2584	2267	+317	+63	+254
82.4	73.0	+9.4	34.0	18.4	+15.6	2584	2190	+394	+98	+296
20.6	18.7	+1.9	8.5	4.5	+ 4.0	646	739	− 93	+20	−113
20.6	19.4	+1.2	8.5	5.5	+ 3.0	646	829	−183	+14	−197
20.6	24.4	−3.8	8.5	15.4	− 6.9	646	1320	−674	−30	−644

toplasmic proportion, whereas potassium was lost in amounts significantly in excess of nitrogen. The decline in urinary sodium excretion ordinarily beginning 24 hours after the withdrawal of insulin and persisting until the termination of the deprivation experiments failed to take place; rather, an increase in urinary sodium excretion occurred with the resultant development of a strongly negative sodium balance. The chloride, calcium and magnesium balances exhibited a premature swing from positive to negative.

(c) *Renal clearance studies.* The renal clearance rates of creatinine, sodium, and potassium are presented in Table IV and those of total ketones, acetoacetic and beta hydroxybutyric acids in Table V. During the administration of ACTH the endogenous creatinine clearance and the potassium clearance increased significantly whereas the renal clearance of sodium remained essentially unchanged. The clearance rate of acetoacetic acid increased somewhat and that of beta hydroxybutyric acid increased markedly. These increases in ketone clearance are, in all probability, responsible for the comparatively small rise in blood ketone levels which occurred during the administration of ACTH. A large increment in urinary ketone excretion occurred during this period indicating that ketosis was, in fact, substantially increased.

B. Response of the Adrenalectomized-Depancreatized Dog to Insulin Deprivation

1. *Clinical course.*

In some respects, the clinical course of the depancreatized-adrenalectomized dog following insulin withdrawal was milder than that of the depancreatized animal. Although vomiting began earlier (during the second experimental period), the animal did not become anorexic and appeared less prostrated at the end of the experiment than did depancreatized animals subjected to insulin deprivation for the same length of time.

On the other hand, tachycardia became alarming during the third period and was associated with an upward trend in the hemoglobin, hematocrit, and the blood level of non-protein nitrogen.[4] It

[4] The rapidity with which these findings, which were regarded as indications of adrenal insufficiency, appeared occasioned some surprise. It will be recalled that in depancreatized dogs the stress of insulin deprivation was not accompanied by an increased secretion of cortical hormones, as gauged by eosinopenia or increased corticosteroid excretion, until 36 to 48 hours had elapsed following the withdrawal of insulin. One possible explanation for the

TABLE IV

Renal clearances of creatinine, sodium and potassium as related to the eosinophil count preceding and accompanying the administration of ACTH to a depancreatized dog

Period	Creatinine clearance (GFR)	Sodium clearance	Potassium clearance	$\frac{\text{K clearance} \times 100}{\text{GFR}}$	Eosinophil count at end of period
	cc. per min.	*cc. per min.*	*cc. per min.*		*Cells/mm.*^{3}
C I	64	0.60	7	12	—
II	52	0.59	6	12	594
E I	60	0.74	20	32	538
II	73	0.54	12	16	562
III	90	0.74	22	24	94

TABLE V

Renal clearances of total ketones, acetoacetic and beta hydroxybutyric acids preceding and accompanying the administration of ACTH to a depancreatized dog

Period	Creatinine clearance (GFR)	Total ketone clearance	Aceto-acetic acid clearance	Beta hydroxy-butyric acid clearance
	cc./min.	*cc./min.*	*cc./min.*	*cc./min.*
C I	64	2	4	1
II	52	1	2	1
E I	60	3	5	2
II	73	10	10	10
III	90	19	13	32

was felt that the repeated withdrawal of blood for chemical analysis was hastening circulatory collapse, and the animal was therefore transfused with 250 cc. of blood at the end of 36 hours of insulin deprivation. It was calculated that this volume of blood would replace that withdrawn during the control period and the previous experimental periods plus that to be withdrawn during the remainder of the experiment. No further rise in hemoglobin, hematocrit, non-protein nitrogen nor pulse rate occurred following the transfusion, and the clinical condition of the animal improved.

earlier collapse of the adrenalectomized animal is that desoxycorticosterone and cortisone, despite their ability to restore normal vigor, normal serum electrolytes, and a normal insulin requirement did not constitute complete hormonal replacement in the dosage administered.

2. *General metabolic measurements.*

(a) *Blood, plasma, and serum constituents.* The changes in blood, plasma and serum constituents which occurred during this experiment are summarized in Table VII. It is worthy of note that both eosinophil and lymphocyte counts began to fall at the end of 36 hours of insulin deprivation and continued to decline until the end of the experiment. There was, however, no reciprocal elevation in the neutrophil count; all types of leukocytes underwent a simultaneous and proportional decline.

Except for the early rise in indices of hemoconcentration the overall trends in blood constituents differ in only one respect from those observed in deprivation experiments upon depancreatized animals. The decline in serum CO_2 content which occurred during the periods immediately subse-

TABLE VI

The food intakes of an adrenalectomized-depancreatized dog (Lady) and of a depancreatized dog (Frisky) before and after the withdrawal of insulin, together with net balances of nitrogen (corrected for changes in blood NPN), phosphorus and potassium

A. Adrenalectomized-depancreatized dog (body weight 18.4 Kg.)

Period	N_2 ingested	N_2 lost in vomitus	N_2 retained	% feeding retained	N_2 balance (*Gm.*) Gross	N_2 balance (*Gm.*) Net‡	P balance	K balance
	Gm.	*Gm.*	*Gm.*				*mg.*	*mEq.*
C I*	11.9	0	11.9	100	+1.7	+0.9	+ 98	+ 8.8
II*	11.9	0	11.9	100	+1.2	+1.2	+ 87	+ 6.6
E I	11.9	0	11.9	100	−1.2	−2.4	− 2	−12.3
II	11.9	2.9†	9.0	76	−2.1	−3.4	− 21	+18.6
III	11.9	4.1	7.8	66	−2.1	−1.8	+ 28	+ 8.5
IV	11.9	13.4†	0	0	−4.9	−5.2	−341	− 2.5
V	11.9	5.2	6.7	56	−5.4	−3.4	−302	− 1.8

B. Depancreatized dog (body weight 17.1 Kg.)

C I*	10.1	0	10.1	100	+1.9	+2.5	+ 35	− 0.4
II*	10.1	0	10.1	100	−0.3	+0.2	−110	− 2.7
E I	10.1	0	10.1	100	−1.1	−1.4	−268	−21.9
II	10.1	0	10.1	100	−2.0	−1.9	−157	− 1.7
III	7.8	0	7.8	77	−2.5	−2.3	−253	− 5.2
IV	0.4	0.4	0	0	−8.1	−7.9	−579	−30.1
V	4.9	3.2	1.7	35	−7.3	−8.1	−522	−44.0

* Data for control periods are expressed as mean values per 12-hour period.
† Vomitus contaminated with urine.
‡ Corrected for changes in blood non-protein nitrogen by assuming body water to be 53 per cent of body weight and non-protein nitrogen to be distributed uniformly therein.

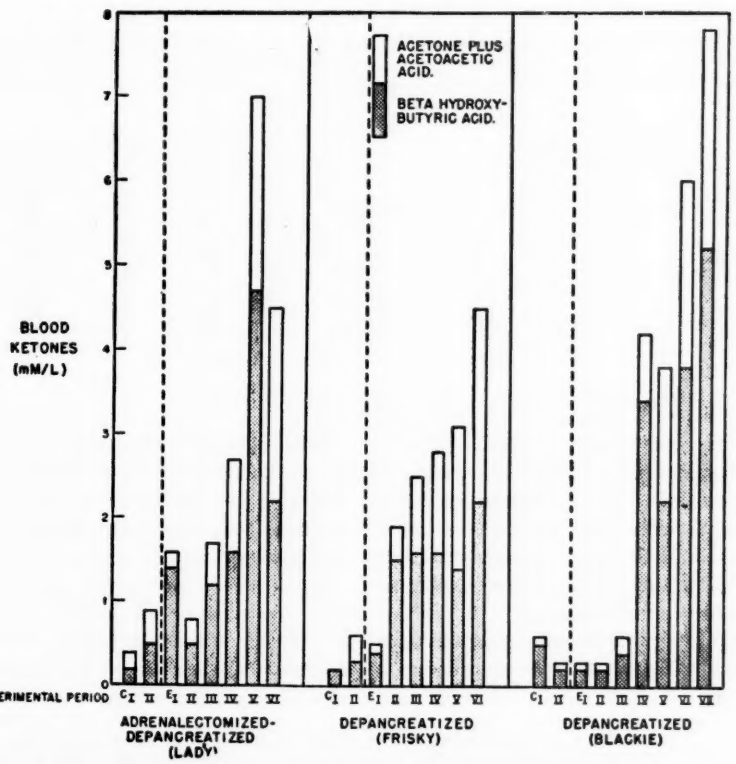

FIG. 2. CHANGES IN THE LEVELS OF BLOOD KETONES BEFORE AND AFTER THE WITHDRAWAL OF INSULIN FROM AN ADRENALECTOMIZED-DEPANCREATIZED DOG AND FROM TWO DEPANCREATIZED ADRENALLY-INTACT ANIMALS

quent to insulin withdrawal was eventually arrested and ultimately reversed. It seems probable that this reversal was due to the early and repeated vomiting, and the disproportionate loss of chloride with respect to sodium which it entailed.

The serum was observed to become lipemic (chylomicron counts and lipokrits were not determined in this study) after only 12 hours of insulin deprivation; thereafter, lipemia rapidly increased. Ketonemia was unequivocal after 36 hours of deprivation and ultimately equalled that observed in depancreatized dogs during terminal acidosis (Figure 2).

(b) *Metabolic balances.* The metabolic balance data is presented in detail in Table VIII and in summary form in Table IX. In Figure 3 the balances of nitrogen, potassium and phosphorus are shown graphically. Although the negativity of both nitrogen and phosphorus balances increased considerably during the last 24 hours of insulin deprivation, the net losses of these substances were much smaller than those observed during comparable periods of deprivation in depancreatized dogs whose adrenals were intact (1). Potassium was lost in excess of nitrogen only during the first 12 hours following insulin withdrawal, the loss thereafter being negligible. Magnesium remained in equilibrium until the final experimental period, when the urinary excretion rate increased sufficiently to render the balance negative. The calcium balance, initially positive, became negative as insulin deprivation continued.

The severe glycosuria which occurred upon the withdrawal of insulin was accompanied initially by heavy losses of sodium and chloride; thereafter, urinary sodium and chloride were well conserved. The external balance of chloride became negative only terminally as vomiting increased.

TABLE VII

Blood, plasma, and serum constituents before and after the withdrawal of insulin from an adrenalectomized-depancreatized dog

Date and time	Beginning of period	Body weight	Pulse	Total WBC	Neutrophils	Lymphocytes	Eosinophils	Sugar	CO_2 content	Total protein
		Kg.			*Cells/mm.³*			*mg. %*	*mEq.*	*Gm. %*
February 20—am	C I	18.243	—	11,000	7,337	2,420	662	201	27.3	6.6
23—am	C II	18.356	68	7,300	4,869	1,825	438	232	29.4	6.9
26—am	E I*	18.427	64	10,500	6,972	2,520	506	253	29.5	5.9
26—pm	E II	18.158	76	10,640	6,937	2,554	613	305	23.8	5.8
27—am	E III†	18.215	140	9,250	5,791	2,627	582	325	17.4	5.2
27—pm	E IV	18.356	160	10,800	8,014	1,901	413	364	16.9	5.8
28—am	E V	17.761	120	8,200	5,789	1,492	419	392	22.6	5.7
28—pm	E VI	17.414	140	6,400	4,301	1,408	259	296	23.3	5.0

* Insulin withdrawn.
† 250 cc. blood transfusion.

(c) *Renal clearance studies.* The renal clearance rates of creatinine, sodium, and potassium are presented in Table X. A pronounced fall in the glomerular filtration rate occurred within 36 hours after insulin deprivation; no further decline occurred thereafter, due perhaps to the blood transfusion administered at this point.

The trends in both sodium and potassium clearance resemble those observed following the withdrawal of insulin from depancreatized dogs; and, as in the adrenally-intact animals, a decreased tubular reabsorption (or an increased tubular secretion) of potassium apparently occurred in the terminal period.

DISCUSSION

Twelve hours' artificial stimulation of the adrenal cortex with ACTH in an animal previ-

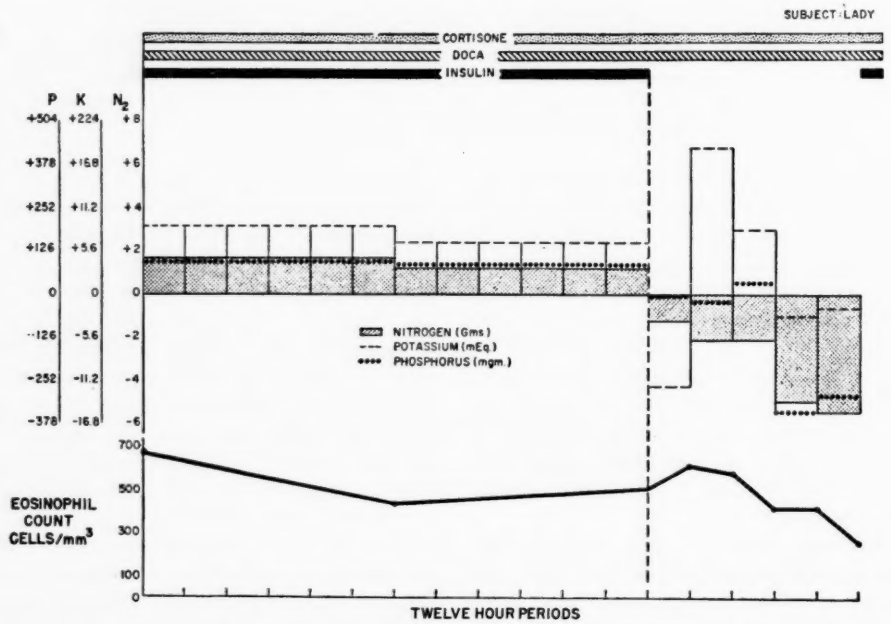

FIG. 3. METABOLIC BALANCES OF NITROGEN, POTASSIUM AND PHOSPHORUS AS RELATED TO THE EOSINOPHIL COUNT BEFORE AND AFTER THE WITHDRAWAL OF INSULIN FROM A DEPANCREATIZED-ADRENALECTOMIZED DOG

TABLE VII—*Continued*

Blood, plasma, and serum constituents before and after the withdrawal of insulin from an adrenalectomized-depancreatized dog

Hgb.	Hct.	NPN	Creatinine	Total ketones	Beta hydroxybutyric acid	Acetoacetic acid	Na	Cl	K	Ca	P	Mg
Gm. %	*%*	*mg. %*	*mg. %*		*mm./L.*		*mEq./L.*	*mEq./L.*	*mEq./L.*	*mg. %*	*mg. %*	*mEq./L.*
14.9	47.6	27	0.33	0.4	0.2	0.2	150.0	104.2	4.0	9.2	2.6	1.2
15.5	50.3	35	0.33	0.9	0.5	0.4	138.0	99.0	4.0	—	2.4	1.3
15.7	49.2	35	0.33	1.6	1.4	0.2	142.4	102.5	4.0	9.4	2.5	1.2
16.3	50.8	47	0.43	0.8	0.5	0.3	139.0	104.0	4.0	8.4	4.0	1.2
16.8	52.3	60	0.54	1.7	1.2	0.5	134.4	98.2	4.4	6.9	4.4	1.5
17.0	50.0	57	0.82	2.7	1.6	1.1	126.0	92.0	4.4	7.1	5.2	—
15.6	47.0	60	0.87	7.0	4.7	2.3	129.0	87.4	4.3	6.8	4.8	2.0
17.0	51.5	40	1.06	4.5	2.2	2.3	133.6	81.4	3.7	6.2	4.7	1.8

ously deprived of insulin for only 24 hours resulted in a premature exacerbation of the diabetic state and in the appearance of a complex of metabolic events ordinarily observed to occur spontaneously only after 48 to 60 hours of insulin deprivation. Events common to pure insulin-deprivation acidosis and ACTH-accelerated acidosis include the following: (a) Increased catabolism of protoplasm; (b) loss of potassium in excess of nitrogen; and (c) increased ketosis. In view of the limitations of the eosinophil count as an index of adrenal cortical activation it is surprising that the use of this index for determination of ACTH dosage resulted in metabolic alterations which were similar not only in direction but in magnitude to those observed in pure insulin-deprivation acidosis.

In most respects, the effects produced by ACTH under the condition of this experiment resemble those previously described in non-diabetic individuals (2–8). Net losses of nitrogen, excess potassium, phosphorus, and calcium resulted from administration of the hormone and the glomerular filtration rate, as estimated from the endogenous creatinine clearance, was increased. The ketosis and the vomiting resulting from the animal's accelerated progress toward diabetic acidosis appeared to introduce a number of modifications in the metabolic pattern ordinarily resulting from ACTH administration. Thus, negative rather than positive balances of sodium and chloride were obtained. Obligatory excretion of sodium in combination with the ketone bodies took place, and large amounts of chloride were lost in the vomitus. Magnesium was also lost, a result in disagreement with that reported by other investigators (6) in a non-diabetic individual. Whether this loss reflects a general drain on fixed bases imposed by the ketonuria, or some other mechanism, is not clear. With respect to ketone metabolism the effects produced by ACTH agree with those reported in diabetic patients and animals by several other investigators (9–12).

Except for the rise in the glomerular filtration rate and the increase in the rate of sodium excretion, the metabolic pattern resulting from ACTH administration was virtually identical with that previously found to occur 24 hours later in this animal during the terminal phase of insulin-deprivation acidosis. In the absence of pertinent data, it seems reasonable to assume that the dehydration from which the animal suffered during the final phases of the insulin deprivation experiments modified the renal response to ACTH. It can, indeed, be argued that had ACTH stimulation not occurred in this critical situation the glomerular filtration rate might have fallen further. The increase in sodium excretion seems logically attributable to the suddenness with which severe ketosis developed during ACTH treatment, the renal ammonia-producing mechanism being thereby denied an opportunity to become fully activated.

In fulfilling the first postulate, the results of this experiment are compatible with the hypothesis that increased adrenal cortical activity is a factor conditioning the occurrence of the following components of acidosis resulting from insulin deprivation: (a) Increased catabolism of protoplasm; (b) loss of potassium in excess of nitrogen; and (c) increased ketosis. However, these results alone do not suffice to establish the correctness of the hypothesis and an examination of the manner in

TABLE VIII

Metabolic balance data before and after the withdrawal of insulin from an adrenalectomized-depancreatized dog

Date	Period	INTAKE							OUTPUT									
									Urine									
		N_2	Na	Cl	K	Ca	Mg	P	Vol.	N_2	Na	Cl	K	Ca	Mg	P	Glucose	Creatinine
		Gm.	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mg.*	*cc.*	*Gm.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mg.*	*Gm.*	*mg.*
February 20—am 23—am	C I	71.4	182.4	392.4	387.6	139.2	27.6	4747	3747	56.4	148.3	370.9	335.0	3.5	1.4	3035	42.8	1499
February 23—am 26—am	C II	71.4	182.4	392.4	387.6	139.2	27.6	4747	4085	59.3	148.6	351.3	348.0	3.5	1.8	3103	81.7	1420
February 26—am pm	E I	11.9	30.4	65.4	64.6	23.2	4.6	791	2067	12.2	55.8	62.0	76.9	0.4	1.1	631	88.4	272
February 26—pm 27—am	E II	11.9	30.4	65.4	64.6	23.2	4.6	791	1106	9.8	7.2	16.1	42.4	0.3	1.0	424	65.1	220
February 27—am pm	E III	11.9	30.4	65.4	64.6	23.2	4.6	791	979	9.0	0.8	0.7	42.7	0.1	0.1	426	51.6	217
February 27—pm 28—am	E IV	11.9	30.4	65.4	64.6	23.2	4.6	791	257	2.5	0.3	0.0	8.4	0.0	0.4	173	8.3	76
February 28—am pm	E V	11.9	30.4	65.4	64.6	23.2	4.6	791	1230	11.2	5.0	0.2	56.3	0.2	3.3	646	48.7	312

TABLE VIII—*Continued*

Metabolic balance data before and after the withdrawal of insulin from an adrenalectomized-depancreatized dog

Date	Period	OUTPUT																							
		Urine plus vomitus									Vomitus								Feces						
		Vol.	N_2	Na	Cl	K	Ca	Mg	P	Glucose	Vol.	N_2	Na	Cl	K	Ca	Mg	P	N_2	Na	Cl	K	Ca	Mg	P
		cc.	*Gm.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mg.*	*Gm.*	*cc.*	*Gm.*	*Eq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mg.*	*Gm.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mEq.*	*mg.*
February 20—am 23—am	C I	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	10.0	0	0	0	199	20.9	1600
February 23—am 26—am	C II	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
February 26—am pm	E I	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
February 26—pm 27—am	E II	285	3.3	7.1	11.4	3.6	1.3	1.0	237	12.4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
February 27—am pm	E III	—	—	—	—	—	—	—	—	—	670	4.1	26.8	61.6	13.4	11.0	1.2	271	4.7	0	0	0	93.2	13.7	618
February 27—pm 28—am	E IV	1150	6.5	0	20.7	29.9	4.9	0.2	503	37.8	710	6.9	21.0	65.0	28.8	12.6	2.8	448	—	—	—	—	—	—	—
February 28—am pm	E V	—	—	—	—	—	—	—	—	—	1440	5.2	25.9	93.6	10.0	10.5	3.6	378	—	—	—	—	—	—	—

which the more rigorous demands of the second postulate are fulfilled is required.

Components of pure insulin-deprivation acidosis which were diminished or suppressed in the adrenalectomized-depancreatized animal maintained on a fixed dose of adrenal cortical hormones during insulin deprivation and which, in fulfilling the second postulate, appear to qualify as adrenal-conditioned, include the following: (a) Hematologic changes comprising increased neutrophil and total white blood cell counts and markedly decreased eosinophil and lymphocyte counts; (b) increased catabolism of protoplasm; (c) loss of potassium in excess of nitrogen; and (d) decreased sensitivity to injected insulin.

Both qualitative and quantitative differences obtained between the hematologic responses of the adrenalectomized-depancreatized and the depancreatized dogs to insulin deprivation. The neutrophil and total white blood cell counts showed a decrease rather than an increase in the doubly-operated animal, and the decrease in the eosinophil and lymphocyte counts was less marked in the adrenalectomized than in the adrenally-intact dog. These observations are in accord with those reported by Muehrcke, Staple, and Kark (13) and Herlant (14). In the light of the meager knowledge currently available regarding the factors which control the levels of the various circulating leukocytes, less interest attaches to the differences between the responses of the two types of animals than to their similarities. The fact that both eosinopenia and lymphopenia occurred to some extent in an adrenalectomized animal maintained on a constant supply of adrenal cortical hormones during stress agrees with the concept of Selye (15) and Engel (16) that these hematologic reactions may be more correctly viewed as conditioned responses than as pure effects of adrenal hormone action.

The finding that less protoplasm was catabolized by the adrenalectomized-depancreatized than by the depancreatized animals during the stress of insulin deprivation is in harmony with many previous reports (17–24). The adrenalectomized animal's failure to excrete excess potassium seems logically attributable to a failure of cellular potassium release; inasmuch as the level of serum potassium remained stationary throughout, the conservation of potassium cannot be ascribed to impaired renal excretion resulting from adrenal insufficiency. The observation that stress increased the insulin sensitivity of the adrenalectomized dog is in accord with the finding of de Bodo, Sinkoff, and Kiang (25) that insulin sensitivity is enhanced in adrenalectomized dogs suffering from adrenal insufficiency following the prolonged deprivation of desoxycorticosterone.

Although the finding that ACTH administration increased ketosis in the depancreatized adrenally-intact animal suggested that ketosis is an adrenal-conditioned event, this expectation was not borne out in the experiment on the adrenalectomized-depancreatized animal. The finding that the level of blood ketones attained by the adrenalectomized-depancreatized dog at the conclusion of the period of insulin deprivation did not differ materially from that previously observed in depancreatized animals is, however, in accord with a number of observations made by other investigators. The ability of anterior pituitary extracts to elevate the levels of blood ketones to the same extent in adrenalectomized animals as in adrenally-intact animals has been clearly demonstrated (26–29). Ketonuria has often been noted to be minimal in adrenalectomized animals treated with ketogenic extracts, giving rise to the impression that ketogenesis has not been stimulated. However, studies comprising determinations of blood as well as urine ketone levels have shown that the blood levels attained differ little from those observed in intact animals (30).

In view of the results indicating that increased adrenal activity is of little or no significance in the genesis of ketonemia, it is not surprising that the same conclusion is suggested regarding changes in the concentration of blood fats. In the adrenalectomized-depancreatized dog lipemia became visible after only 12 hours of insulin deprivation; in the depancreatized animals lipemia did not become evident until a somewhat longer time had elapsed after the withdrawal of insulin and it was less marked. If, as is suggested by a number of studies (31, 32), the corticosteroids participate significantly in fat mobilization, the concentration of cortical hormones required to condition this reaction must be comparatively low.

The adrenalectomized-depancreatized dog proved capable of conserving sodium and chloride as quickly and efficiently as did the depancreatized

TABLE IX

Summary of balance data presented in Table VIII

	Nitrogen (*Gm.*)			Sodium (*mEq.*)			Chloride (*mEq.*)			Potassium (*mEq.*)		
Period	Intake	Output	Balance	Intake	Output	Balance	Intake	Output	Balance	Intake	Output	Balance
C* I	11.9	10.2	+1.7	30.4	24.7	+ 5.7	65.4	61.8	+ 3.6	64.6	55.8	+ 8.8
II	11.9	10.7	+1.2	30.4	24.8	+ 5.6	65.4	58.5	+ 6.9	64.6	58.0	+ 6.6
E I	11.9	13.1	−1.2	30.4	55.8	−25.4	65.4	62.0	+ 3.4	64.6	76.9	−12.3
II	11.9	14.0	−2.1	30.4	14.3	+16.1	65.4	27.5	+37.9	64.6	46.0	+18.6
III	11.9	14.0	−2.1	30.4	27.6	+ 2.8	65.4	62.3	+ 3.1	64.6	56.1	+ 8.5
IV	11.9	16.8	−4.9	30.4	21.3	+ 9.1	65.4	85.7	−20.3	64.6	67.1	− 2.5
V	11.9	17.3	−5.4	30.4	30.9	− 0.5	65.4	93.8	−28.4	64.6	66.4	− 1.8

* Aggregate data for control periods are here expressed as mean values per 12-hour period, for readier comparison with the 12-hour experimental periods.

animal. Moreover, the renal tubular absorption of potassium decreased (or the tubular secretion increased) terminally in the adrenalectomized as well as in adrenally-intact animals. These findings agree with the observation (33) that bilaterally adrenalectomized patients are able to reduce sodium excretion when subjected to salt depletion, and with that of Ingle, Prestrud, Li, and Evans (34) that adrenalectomized rats maintained on a constant intake of adrenal cortical hormones are capable of "physiologically economical adaptations in sodium, chloride and potassium balance." They suggest that intrinsic renal mechanisms, rather than hormonal influences, predominate in the control of electrolyte excretion.

The concept that an exacerbation of the diabetic state is a logical result of stress in the organism with defective pancreatic islet cell function is supported by general physiologic considerations. An increased need for carbohydrate may be the first effect of stress in general, as Engel (35) points out, since stress regularly results in hypoglycemia and death in adrenalectomized or hypophysectomized animals in which the activation of the adrenal cortex and increased protein catabolism which follow stress in intact animals do not occur. As stores of carbohydrate begin to be depleted, the counteraction of increased protein catabolism, gluconeogenesis and decreased insulin sensitivity takes place in the presence of a normally responsive adrenal cortex. In the diabetic organism, preserved from carbohydrate starvation only by exogenous insulin, stress-induced activation of these adrenal-conditioned mechanisms fails of its physiologic purpose. Stores of preformed carbohydrate are only partially utilized due to decreasing effectiveness of injected insulin and together with newformed carbohydrate are excreted in the urine, carrying with them water and electrolytes.

TABLE X

Renal clearances of creatinine, sodium, and potassium before and after the withdrawal of insulin from an adrenalectomized-depancreatized dog

Period	Creatinine clearance (GFR)	Sodium clearance	Potassium clearance	K clearance ×100 / GFR
	cc./min.	*cc./min.*	*cc./min.*	
C I	105	0.23	19	18
II	100	0.24	20	20
E I	99	0.54	26	26
II	—	—	—	—
III	44	0.01	13	30
IV	—	—	—	—
V	45	0.05	19	43

The studies of Long and Lukens (36) demonstrated that an intact pituitary-adrenal axis is essential for full manifestation of pancreatic diabetes. The present studies suggest that the adrenal cortical hormones, in conditioning a number of the components of the non-specific alarm reaction to the stress of insulin deprivation, may contribute significantly to the pathologic physiology of experimental diabetic acidosis as well.

SUMMARY

1. The metabolic changes which characterized the stressful terminal phases of insulin deprivation in the depancreatized dog have been compared with: (a) The changes which accompany the induction of a comparable degree of eosinopenia by

TABLE IX—*Continued*

Summary of balance data presented in Table VIII

Calcium (*mEq.*)			Magnesium (*mEq.*)			Phosphorus (*mg.*)				
Intake	Output	Balance	Intake	Output	Balance	Intake	Output	Balance	Theoretical based on Ca	Protoplasmic balance (total P minus P based on Ca)
23.2	17.2	+ 6.0	4.6	2.0	+2.6	791	639	+152	+ 54	+ 98
23.2	17.2	+ 6.0	4.6	2.0	+2.6	791	650	+141	+ 54	+ 87
23.2	19.0	+ 4.2	4.6	3.8	+0.8	791	755	+ 36	+ 38	− 2
23.2	20.2	+ 3.0	4.6	4.7	−0.1	791	785	+ 6	+ 27	− 21
23.2	29.7	− 6.5	4.6	4.0	+0.6	791	821	− 30	− 58	+ 28
23.2	36.1	−12.9	4.6	6.1	−1.5	791	1248	−457	−116	−341
23.2	29.3	− 6.1	4.6	9.6	−5.0	791	1148	−357	− 55	−302

the administration of ACTH to a depancreatized dog deprived of insulin for a comparatively brief period; and (b) the changes which accompany the withdrawal of insulin from an adrenalectomized-depancreatized dog maintained upon a fixed dose of adrenal cortical hormones.

2. The administration of ACTH under these circumstances was found to result in a premature exacerbation of the diabetic state and in the appearance of a complex of metabolic phenomena characteristic of experimental diabetic acidosis resulting from prolonged insulin deprivation.

3. The withdrawal of insulin from an adrenalectomized-depancreatized dog maintained on a fixed dose of adrenal cortical hormones resulted in diminution or suppression of a number of components of pure insulin-deprivation acidosis.

4. It is concluded that those constituents of pure insulin deprivation acidosis which are: (a) Temporally related to the occurrence of evidences of increased adrenal activity; (b) prematurely induced by exogenous stimulation of the adrenal cortex during mild stress; and (c) diminished or suppressed by maintenance of a fixed supply of adrenal cortical hormones during severe stress are, in fact, adrenal-conditioned.

These constituents include: (1) Hematologic changes comprising increased neutrophil and total white blood cell counts and markedly decreased eosinophil and lymphocyte counts; (2) increased catabolism of protoplasm; (3) loss of potassium in excess of nitrogen; and (4) decreased sensitivity to injected insulin.

5. It would appear that the increase in adrenal cortical activity which occurs in response to the stress of insulin deprivation, by conditioning or sustaining a number of the other constituents of the alarm reaction to this stress, contributes significantly to the pathologic physiology of experimental diabetic acidosis.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to Dr. Fred E. Houghton of Ciba Pharmaceutical Products and Drs. J. M. Carlisle, Augustus Gibson and Elmer Alpert of Merck and Company for generous gifts of steroid hormones, and to Mrs. Kirsten Van Loon for assistance in making the chemical determinations.

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KINETICS OF ELIMINATION OF GLUCOSE FROM THE BLOOD DURING AND AFTER A CONTINUOUS INTRAVENOUS INJECTION

By S. G. JOKIPII and OSMO TURPEINEN [1]

(From the Third Department of Medicine, Helsinki University, Helsinki, Finland)

(Submitted for publication May 12, 1953; accepted November 4, 1953)

The glucose tolerance tests in routine clinical use are predominantly of the oral type, in which the sugar is administered by mouth. Such tests, however, suffer from the disadvantage that the rate of intestinal absorption has an effect on the results, and its variations may entirely overshadow any possible changes in carbohydrate metabolism. This difficulty may, of course, be obviated by administering the sugar by vein, and intravenous tolerance tests have actually been used by many workers (1–8).[2] In these tests the sugar has generally been given as a single rapid injection. While such a method undoubtedly gives useful information, it can hardly be regarded as a quite physiological one. A more natural procedure would be to inject glucose slowly over a long period of time, thus imitating the normal process of absorption. So far, tolerance tests of this type have apparently received scant attention. As it seemed that this method might provide additional information not easily obtainable from the tests based on a single rapid injection, we decided to carry out the following experiments, in which glucose was administered as a continuous injection of constant rate and of relatively long duration.

METHODS

Our subjects were hospitalized patients. During the experiments they were in the postabsorptive state. Approximately isotonic (5 to 6 per cent) glucose solutions in distilled water were used, and their glucose content was determined by polarimetry.

The sterilized glucose solution was run by gravity from the infusion apparatus into the cubital vein. The rate of infusion was kept as nearly constant as possible. It was checked at frequent intervals by means of a drop-counting device and readjusted if necessary. The infusion rates ranged from 155 to 630 mg. of glucose per minute in different experiments. In a typical experiment the infusion lasted 60 minutes. The blood sugar of the subjects was determined in samples of capillary blood by means of the Hagedorn-Jensen method (9), and the samples were generally drawn 10, 20, 30, 40, 50, and 60 minutes after the start of the infusion and in about one-half of the experiments also 30 and 60 minutes after the end of the infusion. Urine samples were collected at the end of the infusion and often also 30 and 60 minutes later. The subjects remained recumbent during the whole experiment.

RESULTS

Mean blood glucose curve during the infusion

The above-described glucose tolerance tests were carried out in some thirty patients in all. After the exclusion of diabetics and a few other patients suffering from diseases known to affect carbohydrate metabolism, twenty-four patients remained whose carbohydrate metabolism was presumably normal. Their results were averaged, and the course of the mean blood glucose value during the infusion is shown in Figure 1. The mean fasting blood glucose prior to the infusion was 84.9 mg. per dl.[3] This initial basal value was used as the zero level, and the curve shows the mean increases above this basal value after specified time intervals. It is seen that the blood glucose initially rises rapidly, then more and more slowly and seems to be approaching a plateau at the end of the infusion. When the plateau is reached, the rate of elimination obviously equals the rate of infusion.

It is of considerable interest to examine whether the blood glucose curve shown in Figure 1 could be given some relatively simple mathematical interpretation. It is known that certain substances are removed from the blood and other compartments of the body through which they are distributed at a rate defined by a first-order reaction.

[1] Present Address: Department of Physiology, Veterinary College, Helsinki, Finland.

[2] This is not meant to be a complete bibliography. Tunbridge and Allibone (3) list no less than 37 publications dealing with intravenous glucose tolerance tests.

[3] dl. = deciliter = 100 ml.

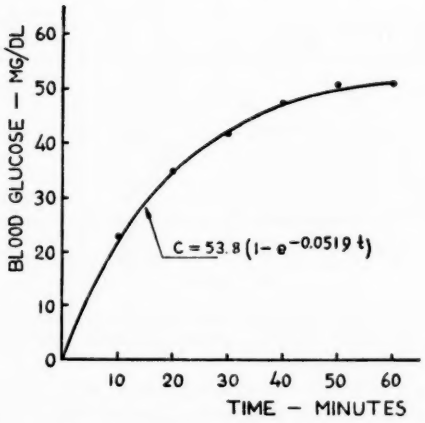

FIG. 1. THE AVERAGE COURSE OF THE BLOOD GLUCOSE VALUE IN TWENTY-FOUR NORMAL SUBJECTS DURING THE INFUSION

The dots indicate the arithmetic means of the observations; the curve represents the equation shown. The mean of the pre-injection blood sugar (the zero level in the graph) was 84.9 mg. per dl., that of the final blood sugar at 60 minutes 135.7 mg. per dl., and the mean rate of infusion 297 mg. per min.

This has been shown to be the case with, *e.g.*, acetone (10), calcium (11), creatinine (12), xylose (13), mannitol (14), and also with glucose under experimental conditions different from ours (5).

In a first-order reaction the amount of material which is eliminated per unit of time is directly proportional to the amount that is present:

$$-\frac{dq}{dt} = kq \qquad (1)$$

(q = quantity of substance in the body, mg; t = time, minutes; k = the specific reaction-rate constant or the velocity constant of elimination, min.$^{-1}$). If the volume of distribution (v dl) of the substance remains constant, the situation may also be described:

$$-\frac{dc}{dt} = kc \qquad (2)$$

(c = concentration of the substance in its volume of distribution, mg. per dl.).

During the infusion the substance is simultaneously added to the body, and the rate of the concentration change caused by this obviously equals the rate of infusion (ρ mg. per min.) divided by the volume of distribution:

$$+\frac{dc}{dt} = \frac{\rho}{v} \qquad (3)$$

Accordingly, the rate of the net increase in the concentration of the substance is as follows:

$$\frac{dc}{dt} = \frac{\rho}{v} - kc \qquad (4)$$

If the concentration of the substance at the start of the infusion is zero (c = 0, t = 0), the equation (*4*) gives on integration:

$$c = \frac{\rho}{vk}(1 - e^{-kt}) \qquad (5)$$

Thus, it is evident that if a substance is injected into the blood at a constant rate (and if the first-order reaction equation holds), the form of the concentration-time curve depends on, besides the rate of injection (ρ), two characteristics of the subject: the velocity constant of elimination (k) and the volume of distribution (v). If these two are known, the curve can easily be drawn. On the other hand, if the curve has been experimentally determined, the unknown quantities k and v can be derived from it.

The above reasoning should also be applicable to the glucose infusions described in this paper. In this case the concentration c naturally represents the blood glucose concentration in excess above the "basal" value at the start of the infusion.

We have found that the data given in Figure 1 can quite satisfactorily be fitted with a curve corresponding to the equation (*5*). The fitting was done graphically on semilogarithmic paper, and the best fit was obtained with the following:

$$c = 53.8(1 - e^{-0.0519t}) \qquad (5a)$$

Thus, the velocity constant (k) was 0.0519 min.$^{-1}$.

The rate of elimination could, of course, also be expressed as the "period of half-life" ($t_{\frac{1}{2}}$) indicating the time required for the "excess" glucose concentration to be reduced by one-half.

$$t_{\frac{1}{2}} = \frac{0.693}{k} \qquad (6)$$

In this case the half-life was 13.4 minutes.

As the mean rate of infusion (ρ = 297 mg. per min.) is known, the equation (*5*) also permits us to compute the mean volume of distribution (v). This was found to be 10.6 liters. As the mean

body weight of the subjects was 62.7 Kg., the mean volume of distribution expressed in relation to body weight was 17.0 per cent. The value of v was also computed as percentage of the "lean body mass" (LBM). The latter, however, was not determined experimentally, but merely calculated from the predicted basal metabolic rate (15). The mean volume of distribution was found to be 20.0 per cent of the LBM.

Mean blood glucose curve after the infusion

In about one-half of the experiments the blood glucose was determined not only during the infusion but also 30 and 60 minutes after the end of the infusion. We had twelve cases in which this was done, and their mean blood glucose curve is shown in Figure 2. This is a curve which first behaves in a way very similar to that in Figure 1 and after the end of the infusion falls first steeply and then more gradually.

The rising part of the curve shown in the Figure 2 was again fitted with a curve corresponding to the equation (*5*) and the best fit was obtained with the following:

$$c = 55.0(1 - e^{-0.0485t}) \qquad (5b)$$

Thus, in this series the value of the velocity constant of elimination was 0.0485 min.$^{-1}$, which corresponds to a half-life of 14.3 minutes.

As the mean rate of infusion was 284 mg. per min., the volume of distribution was found to be 10.6 liters or 17.5 per cent of the mean body weight or 19.8 per cent of the "lean body mass."

The falling part of the blood glucose curve must obviously be computed with the assumption that the elimination also here follows the rate of a first-order reaction. We may return to the equation (*4*) and note that now $\rho = 0$. So this equation becomes identical with the equation (*2*). If we designate the concentration at the zero time (the end of the infusion) as c_o, we obtain on integration:

$$c = c_0 e^{-kt} \qquad (7)$$

Our experimental data indicated that the final basal value of the blood glucose was not identical with the initial basal value. The mean blood glucose at the start of the experiment in this group was 86.5 mg. per dl., and the concentration 60 minutes after the end of the infusion was lower,

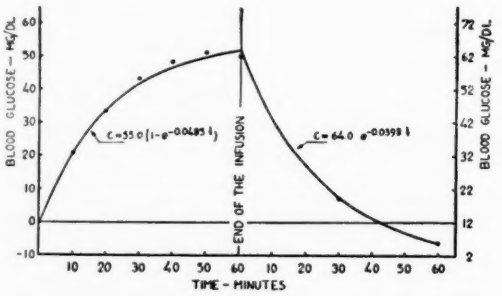

FIG. 2. THE AVERAGE COURSE OF THE BLOOD GLUCOSE VALUE IN TWELVE NORMAL SUBJECTS DURING AND AFTER THE INFUSION

The dots indicate the arithmetic means of the observations; the curves represent the equations shown. The mean of the pre-injection blood sugar was 86.5 mg. per dl., that of the blood sugar at the end of the infusion 136.6 mg. per dl., and 60 minutes after the end of the infusion 80.4 mg. per dl. The mean rate of infusion was 284 mg. per min. The zeros on the axes of ordinates are: on the left—the pre-injection blood sugar; on the right—the calculated limit (74.5 mg. per dl.) which the blood sugar value approaches provided that the fall follows the rate of a first-order equation.

80.4 mg. per dl. (the significance of the difference: P = 0.05 to 0.02), and even this value probably did not yet represent the final basal value.

Our experimental data applied to the equation (*7*) gave the following:

$$c = 64.0\, e^{-0.0398t} \qquad (7a)$$

According to this equation c_o (the glucose concentration at the end of the infusion) was 64 mg. per ml. above the final basal value. However, according to the equation (*5b*) this same concentration (c at 60 minutes) was only 52 mg. per dl. above the initial basal value. The final basal value must, therefore, have been 12 mg. per dl. lower than the initial basal value. The appearance of such a phase of relative hypoglycemia in the latter part of various glucose tolerance tests is, of course, a common observation. It is also worthy of note that the velocity constant of elimination during the latter part of the experiment appeared to be somewhat smaller than during the first part.[4] It seems

[4] The statistical significance of this difference cannot be given accurately, since the values of k were determined through graphical fitting only. From the scatter of the blood glucose values it may, however, be estimated that the standard errors of the values of k were approximately

TABLE I

Data on blood sugar curves treated individually

Patient no.	Age and sex	Diagnosis	p mg./min.	k min.$^{-1}$	v liters	v % of BW†	v % of LBM‡
6	29 M	Emphysema	293	0.065	11.6	17.6	19.6
7	38 M	Rheumatoid arthritis	237	0.041	11.2	18.7	20.5
7*	38 M	Rheumatoid arthritis	436	0.040	13.0	21.0	23.5
9	42 F	Neurocirculatory asthenia	330	0.043	9.2	17.0	20.0
10	54 F	Essential hypertension	628	0.050	11.5	11.2	19.8
11	20 F	Schizophrenia	251	0.076	7.2	13.8	15.4
17	36 M	Duodenal ulcer	277	0.040	13.8	20.3	24.5
45	39 M	Healthy	592	0.063	13.5	19.9	24.0
19	59 M	Acromegaly	365	0.069	11.2	13.5	18.9
22	26 M	Infectious hepatitis	383	0.031	13.2	18.6	20.5
27	30 F	Diabetes mellitus	233	0.052	4.9	9.2	10.7
28	21 M	Diabetes mellitus	327	0.063	5.1	7.3	7.9
29	33 M	Diabetes mellitus	303	0.048	9.4	11.9	13.8

* This experiment was done seven days after the previous one. During this time the patient had gained 2 Kg.
† BW = body weight.
‡ LBM = lean body mass.

as if the dynamic equilibrium of the glucose metabolism were somewhat changed by the test itself.

Individual blood glucose curves

It would obviously be of considerable interest to be able to analyze also the individual data. This, however, is fraught with difficulties, for the individual blood glucose-time curves frequently show irregularities rendering their analysis rather uncertain. In about one-half of the experiments we obtained curves which were regular enough to be fitted with the equation (*5*) with satisfactory precision. The data pertaining to these experiments are given in Table I.

The values of k found by us for individual subjects with presumably normal carbohydrate metabolism ranged from 0.040 to 0.076 min.$^{-1}$. In terms of the half-life these values were equivalent to a range from 17.3 to 9.1 minutes. The range of v was found to be from 7.2 to 13.8 liters or from 11.2 to 21.0 per cent of the body weight or from 15.4 to 24.5 per cent of the "lean body mass."

Among the tests included in Table I are a few done on subjects suffering from diseases known to affect carbohydrate metabolism. The patient No. 19 suffering from acromegaly showed fairly normal values of k and v. The patient No. 22, a case of infectious hepatitis, had a rather low value of k. Of considerable interest are the three diabetics, Nos. *27*, *28*, and *29*.[5] Their values of k were well within the normal range. On the other hand, the values of v were strikingly low, in two patients only about one-half of the normal and in the third also obviously below the normal range.

DISCUSSION

Our experimental data are in good agreement with the hypothesis that the elimination of the exogenous glucose from the blood takes place at the rate of a first-order reaction. In view of the fact that this physiological process is generally regarded as being a rather complex phenomenon, it is of interest to note that its reaction rate is defined by such a relatively simple equation.

Two quantities of physiological significance enter this equation, k and v. k, the velocity constant of elimination, is probably the best single index of the capacity of the organism to eliminate glucose from the blood and from the rest of its volume of distribution. It also has the distinct advantage of being independent of the body size. The velocity constant k indicates the fraction of glucose which is eliminated per unit of time, and its dimension is the reciprocal of time, *e.g.*, min.$^{-1}$. If so preferred, the period of half-life ($t_{\frac{1}{2}}$) can be used instead of k.

v is the total volume of the body fluids through

0.004. Thus the difference of 0.0087 between these two values of k may be somewhat too small to be statistically significant.

[5] All three suffered from moderately severe diabetes and were treated with protamine zinc insulin, which was withheld from them for a period of 48 hours before the test. Their pre-injection blood sugar values ranged from 216 to 347 mg. per dl.

which the injected glucose appears to be initially distributed. The volumes of distribution are customarily expressed as percentages of the body weight, and in terms of this measure our mean value of v was 17.0 per cent. This is in fair agreement with the value 19 per cent reported for glucose by Dominguez (16). Very similar values have also been reported for sucrose (18 per cent) and for inulin (15.7 per cent) by McCance (17), for mannitol (18.3 per cent) by Dominguez, Corcoran, and Page (14), and for radioactive chloride (18.9 per cent) by Moore (18).

In regard to the volume of distribution the body weight does not, however, seem to be a very satisfactory standard of reference. In our material the variation among the individual values of v was more effectively reduced if they were expressed in reference to the "lean body mass" (cf. Table I). A still greater uniformity might have been obtained, if experimentally determined values for LBM had been used instead of those computed from the predicted basal metabolic rates (15).

As to the meaning of the volume of distribution, it may be difficult or impossible to correlate it with any anatomically defined space. The value found by us for it is most closely in agreement with the "chloride space," which is often identified with the extracellular fluid. This could possibly be interpreted as indicating that exogenous glucose is initially distributed through extracellular fluid and in the main excluded from cells.

Perhaps a few words should also be said about the observations concerning diabetics, even though our material is very limited. In regard to the values of k, which appeared to be normal, it should be pointed out that in the diabetic with glycosuria this constant does not have quite the same meaning as in the normal person. In the latter the elimination of glucose takes place through utilization only [6] and k is purely the velocity constant of utilization ($k_{el} = k_u$). In the diabetic organism glucose is eliminated through both utilization and renal excretion, and k is the velocity constant of this combined process ($k_{el} = k_u + k_{ex}$). The velocity constant of utilization only must obviously be smaller than the constant of the combined process. All our diabetic subjects had glycosuria, but because of the lack of adequate quantitative data we were not able to compute the pure velocity constant of utilization in these subjects.[7] The definitely small values of v in the diabetics are an interesting finding for which we are not yet prepared to offer an explanation. Obviously this observation must be confirmed in more extensive experiments.

The clinical application of intravenous glucose tolerance tests, in general, seems to be in a rather unsettled state. Lack of uniformity in both technique and interpretation is prevalent. The criterion of normality most often appears to be the time interval taken for the elevated blood glucose to regain its pre-injection value (3) or some defined relatively low value (19) or, conversely, the value of blood sugar at a certain time after injection (4). It is evident that such criteria are valid under strictly defined experimental conditions only and lack more universal applicability and deeper physiological meaning. Up to now, only a few attempts have been made to interpret the blood glucose-time curves in a theoretically more satisfactory way. Ross (20) used a rather complicated method as he measured the surface area under the curve by a planimeter. Hamilton and Stein (21) described a method by which the blood sugar curve was "straightened out" on semilogarithmic paper and characterized by a single figure indicating the slope of the resulting straight line. However, their use of total blood sugar values instead of values indicating the excess above the basal value made their method mathematically unsound and also difficult to apply. The only adequate mathematical treatment of blood glucose curves seems to be that of Greville (5), who characterized the curves by the use of three constants: k, C, and y_o, of which the first is identical with our

[6] None of our non-diabetic subjects showed glycosuria during or after the tests.

[7] In contrast to our findings, Amatuzio, Stutzman, Vanderbilt, and Nesbitt (22), whose paper appeared shortly after ours had been submitted for publication, obtained a clear-cut difference in the values of k between diabetics and normal persons. This discrepancy could hardly have arisen from the differences in the technique of the test. (They used the single rapid injection method.) It may possibly be due to the fact that their patients did not receive exogenous insulin, whereas ours did. The 48-hour period of insulin deprivation imposed on our patients prior to the tests may not have been sufficiently long to permit a complete disappearance of the effect of protamine zinc insulin.

k, the second is definitely correlated with our v, and the third is the "limiting value" of blood sugar.[8]

In this paper we have tried to treat intravenous blood glucose curves in a way which would be both mathematically defensible and physiologically meaningful. As our material is relatively limited, we have sought support for our conclusions from the work published earlier and have found it to a considerable extent. As far as we are aware, no work with a technique comparable to ours has been reported. On the other hand, a large volume of work on tests based on a single rapid injection appears in the literature. Of course, these two types of tests do not necessarily give concordant results. After the single injection the organism is exposed to unphysiologically high blood sugar concentrations (often in excess of 300 mg. per dl.), which may or may not disturb the normal course of glucose metabolism. The continuous type of injection is free from this objection.

We have examined a number of papers dealing with tolerance tests of the single injection type. Most of the reported data are in good agreement with the assumption that exogenous glucose is eliminated at the rate of a first-order reaction. The equation (7) can then be used for calculating the values of k. We carried out this calculation from some published material and found the following mean values: From Lennox and Bellinger (2): $k = 0.037$; from Lozner, Winkler, Taylor, and Peters (4): $k = 0.045$; from Goldberg and Luft (6): $k = 0.063$; and from Moyer and Womack (8): $k = 0.044$. A "typical response" reported by Tunbridge and Allibone (3) gave: $k = 0.054$.[9] So, in spite of great differences in technique, the values of k calculated from this earlier material are in essential agreement with our mean value for k, 0.052. On the other hand, the values reported by Greville (5) are definitely lower, from 0.018 to 0.039.[10] The values of v are more difficult to obtain from the curves of the single injection tests. They can, however, be computed as suggested by Widmark (10) by the use of the equation: $v = \frac{q}{c_0}$, in which q is the quantity of glucose injected and c_0 is the hypothetical concentration which would exist immediately after the injection if the steady state of diffusion were reached at once. c_0 is obtained through extrapolation from the logarithmically linear part of the curve ignoring the initial high concentrations after the injection. This method, however, is not free from objections. Through such a calculation we obtained from Lennox and Bellinger's (2) material $v = 10.3$ liters, from the data of Lozner, Winkler, Taylor, and Peters (4) $v = 8.1$ liters, and from that of Tunbridge and Allibone (3) $v = 9.2$ liters,[11] values which are not far from ours.

To conclude, we believe that the intravenous glucose tolerance test is a method susceptible of a theoretically sound treatment. As our experience and the evidence obtained from earlier work indicate, the elimination of exogenous glucose takes place at the rate of a first-order reaction, at least with fair approximation. From the blood glucose-time curves two constants, k and v, can be derived, which characterize the course of the curve. k is the specific reaction-rate constant or the velocity constant, a concept widely used in chemical kinetics, and v is the apparent volume through which the injected glucose is initially distributed. The values of k and v probably are in a large degree independent of the experimental details of the test, and it is evident that they can also be determined, though possibly less accurately, by means of the single injection technique. In the interest of a theoretically sound and physiologically meaningful interpretation of the glucose tolerance tests, these two constants would obviously offer definite advantages over the more or less arbitrary criteria now commonly used. Further work is certainly required to establish normal mean values and ranges for them. However, from our experiments reported here it would appear that in normal persons the mean value of k is approximately 0.05 $min.^{-1}$ (corresponding to a half-life of some 14 minutes) and that of v about 10 to 11 liters.

[8] Recently Amatuzio, Stutzman, Vanderbilt, and Nesbitt (22) also used the constant k to characterize the glucose tolerance after a single rapid intravenous injection.

[9] Amatuzio, Stutzman, Vanderbilt, and Nesbitt (22) reported values of k ranging from 0.030 to 0.048.

[10] However, the lower values of k were usually associated with such low values of y_0 as to cast some doubt on the physiological soundness of his interpretation.

[11] From the material published by Amatuzio, Stutzman, Vanderbilt, and Nesbitt (22) we obtained the mean value: $v = 13.7$ liters.

SUMMARY

Glucose solutions were injected intravenously at a constant relatively slow rate during a period of 60 minutes into human subjects. The blood glucose level was determined in samples of capillary blood during and, in part of the experiments, also after the injection.

The experimental results were in good agreement with the hypothesis that the elimination of the exogenous glucose from the blood takes place at the rate of a first-order reaction. The form of the blood glucose-time curve is determined by, besides the rate of injection, two characteristics of the subject: k, the specific reaction-rate constant or the velocity constant of elimination, and v, the apparent initial volume of distribution.

The mean value of k obtained from tests with twenty-four subjects whose carbohydrate metabolism was presumably normal was 0.052 min.$^{-1}$. This corresponds to a period of half-life of 13.4 minutes. The mean value of v was 10.6 liters or 17.0 per cent of the body weight or 20.0 per cent of the estimated "lean body mass."

In three diabetic subjects the values of k were found to be within the normal range, but the values of v were strikingly low.

In the interest of a theoretically sound and physiologically meaningful interpretation of the glucose tolerance tests, the authors advocate the use of the constants k and v as the criteria of such tests.

ACKNOWLEDGMENT

The authors wish to acknowledge their indebtedness to Professor William Kerppola, the Head of the Department, for his permission to carry out these experiments and for the provision of facilities in his Department.

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THE FATE OF P^{32} LABELLED DIISOPROPYLFLUOROPHOSPHONATE IN THE HUMAN BODY AND ITS USE AS A LABELLING AGENT IN THE STUDY OF THE TURNOVER OF BLOOD PLASMA AND RED CELLS

By J. A. COHEN and MIA G. P. J. WARRINGA

(From the Medical Biological Laboratory of the National Defence Research Council T. N. O., Rijswijk, Netherlands)

(Submitted for publication May 12, 1953; accepted October 28, 1953)

In recent years diisopropylfluorophosphonate (DFP) has established itself as a useful drug in such conditions as intestinal and vesical atonia, glaucoma and myasthenia gravis. With the consolidation of its place in therapy the desirability of more precise knowledge of the fate of this compound in the human organism became obvious. In the present study the absorption, the distribution in the blood and the excretion of DFP have been studied after intramuscular injection of the radioactive labelled substance (DFP^{32}) into three patients suffering from myasthenia gravis (two cases) and arteriosclerosis cerebri, respectively, and into two normal persons. There are no theoretical reasons why the metabolic fate of DFP should be different from normal in the patients studied. Indeed, no essential differences were observed between results obtained from the three patients and those from the normal persons. Therefore, all results to be described should be considered as applicable to the metabolism of DFP in the normal human organism.

In the course of this work it became obvious that the phosphorus of DFP^{32} was irreversibly bound to the proteins of blood plasma and red cells and that DFP was exclusively broken down to diisopropylphosphate, a substance which is not further metabolised nor taken up into any normal metabolic pool. Such properties suggested that DFP^{32} might prove to be a useful tool in the study of the turnover of blood plasma proteins and red cells. Preliminary results of the use of DFP^{32} in the determination of the life span of red cells and the half life of a plasma protein will be included in this study.

EXPERIMENTAL METHODS

Synthesis of DFP^{32}. DFP^{32} was prepared from $H_3P^{32}O_4$ purchased from the Atomic Energy Research Establishment at Harwell by a method to be described elsewhere. It was injected intramuscularly as a solute in 1 to 2 ml. of peanut oil.

Radioactivity measurements. In the absorption experiments, samples of 0.1 ml. blood obtained from the fingertip were pipetted into flat counting dishes, which were provided with a snugly fitting piece of filter paper in the bottom. After drying, the samples were counted with a conventional Geiger Müller tube. All other radioactivity measurements were carried out with a G.M. liquid counter (M_6 of 20th Century Electronics). All measurements were related to standards consisting of diisopropylphosphate obtained by alkaline hydrolysis of a sample of DFP^{32} of known quantity and radioactivity.

Treatment of blood, urine, and feces for radioactivity measurements. Ten milliliters of blood obtained by venepuncture were collected in sodium citrate (final concentration 0.76 per cent). Red cells were separated from the plasma and washed thrice in normal saline. Counting was carried out on the hemolysate in distilled water. Samples, diluted if necessary, from 24-hour portions of urine were counted directly in the liquid counter. Feces were collected in aliquots over a period of 24 hours and mixed. Samples of 2 Gm. were suspended in a small volume of water and hydrolyzed during 15 minutes at 100° C. in molar NaOH.

Cholinesterase activity. Cholinesterase activity was measured manometrically at 37° C. according to Ammon (1) using acetylcholine as a substrate.

Paper chromatography. Paper chromatography was carried out on samples of 40 µl. of urine using an ascending method with butanol-acetic acid-water mixtures as solvent. The development period lasted 16 hours at a temperature of 23° C.

Organic phosphates were made visible on the paper by a method which was essentially that of Hanes and Isherwood (2) with slight modifications.

Radioactivity on the paper was assessed by exposing x-ray films in direct contact with the chromatogram in the dark room for 72 hours.

RESULTS

The first series of experiments were performed on three young women. Two of these were treated in the Neurological Clinic of the Leyden Uni-

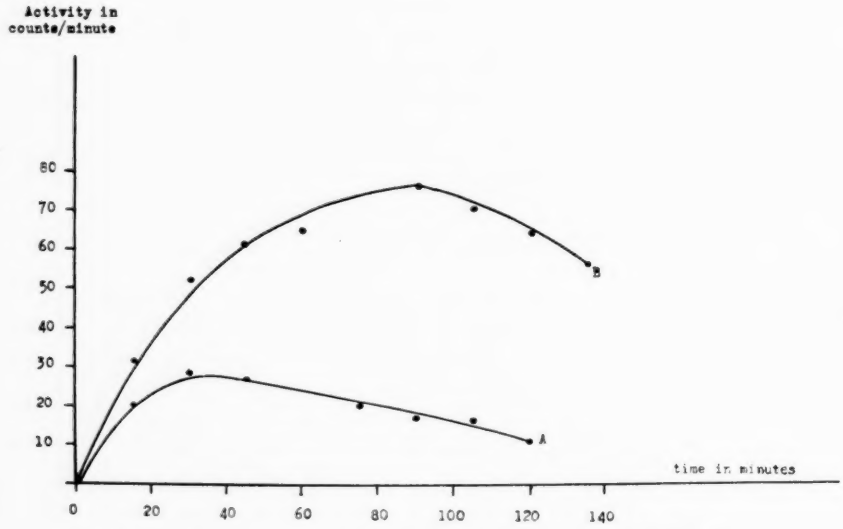


FIG. 1. RADIOACTIVITY IN WHOLE BLOOD AFTER INJECTION OF DFP[32]

Activity expressed in counts per minute per 0.1 ml. sample of blood of patients A and B.

versity Hospital for myasthenia gravis (A and B), the third one (C) was a healthy volunteer. A, B, and C received dosages of 1.990, 0.240, and 0.433 mg. of DFP[32], respectively. The specific activity of the preparation used was approximately 40 μc. per mg. DFP. One of the patients (A) had been treated with OMPA (octamethylpyrophosphoramide) prior to the experiment. At the start of the experiment practically no cholinesterase activity was present in this case in blood plasma and red cells.

After completion of this series it became de-

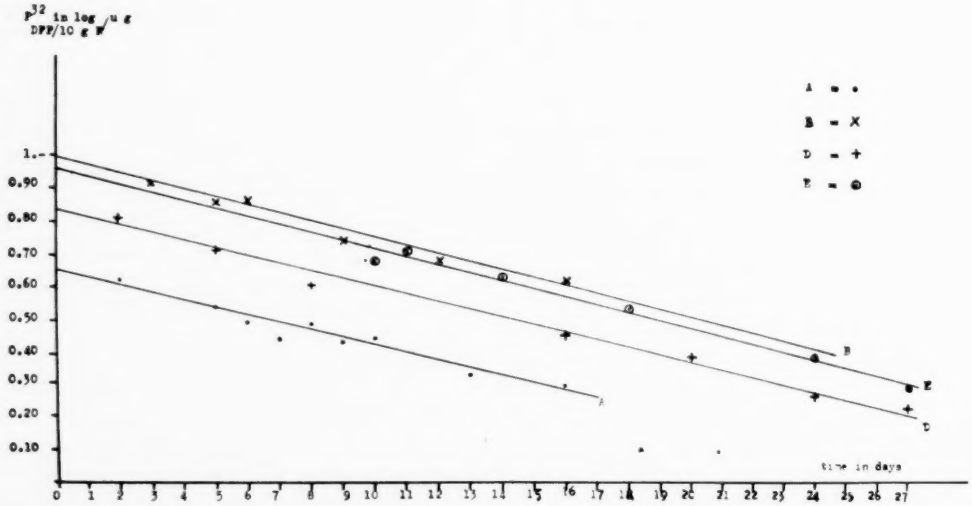


FIG. 2. PLASMA BOUND RADIOACTIVITY AFTER INJECTION OF DFP[32]

Activity expressed as the logarithm of the weight in μg. of DFP bound to 10 Gm. of plasma nitrogen of Subjects A, B, D, and E. Curves drawn by method of least squares.

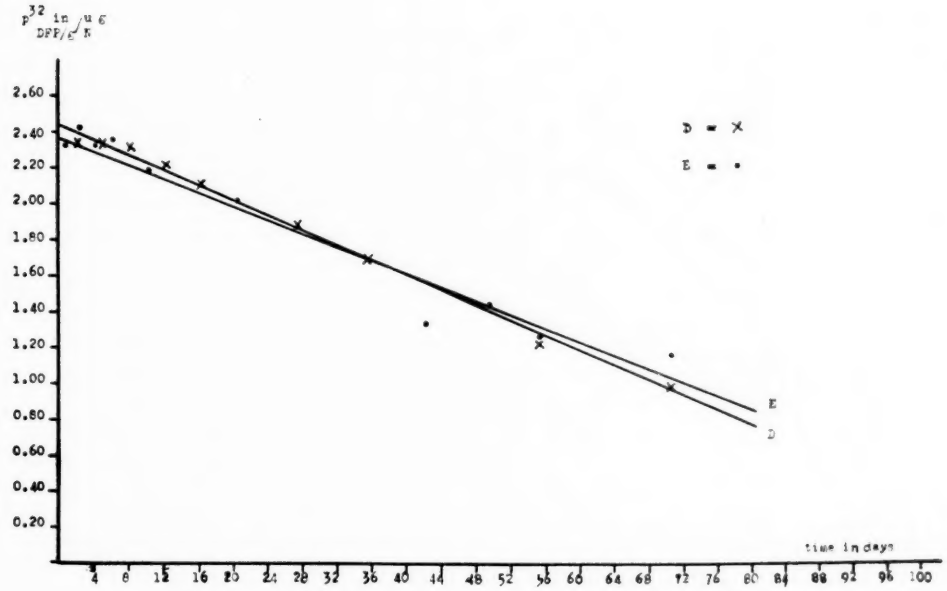


FIG. 3. RED CELL BOUND RADIOACTIVITY AFTER INJECTION OF DFP[32]

Activity expressed in μg. of DFP reacting per Gm. of erythrocyte nitrogen in the blood of Subjects D and E. Curves drawn by method of least squares.

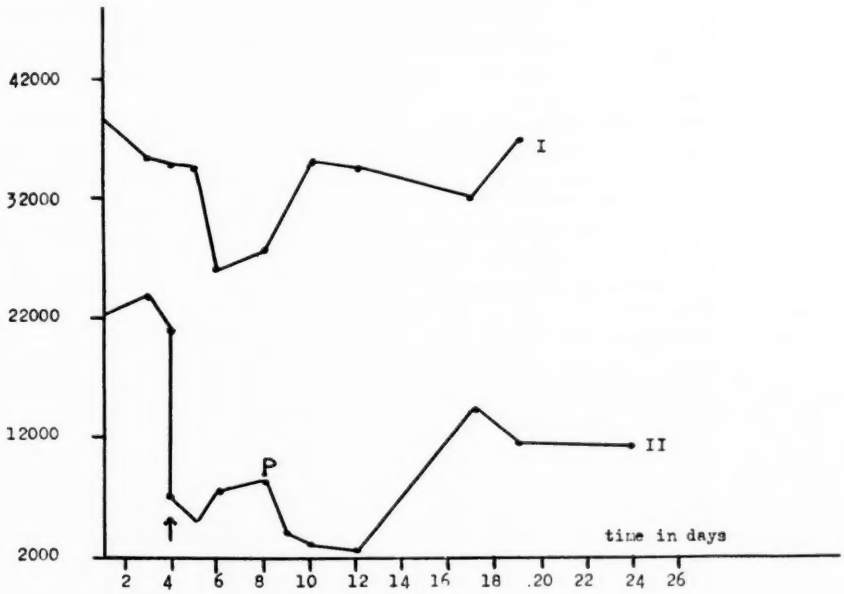


FIG. 4. PLASMA AND RED CELL CHOLINESTERASE

I, Red cell cholinesterase of Case B expressed in μl. CO_2 per hour per 100 Gm. Hb. II, Plasma cholinesterase of same person in μl. CO_2 per hour per 100 Gm. N. The arrow marks the moment of injection of 240 μg. DFP[32]. P indicates the moment of neostigmine injection.

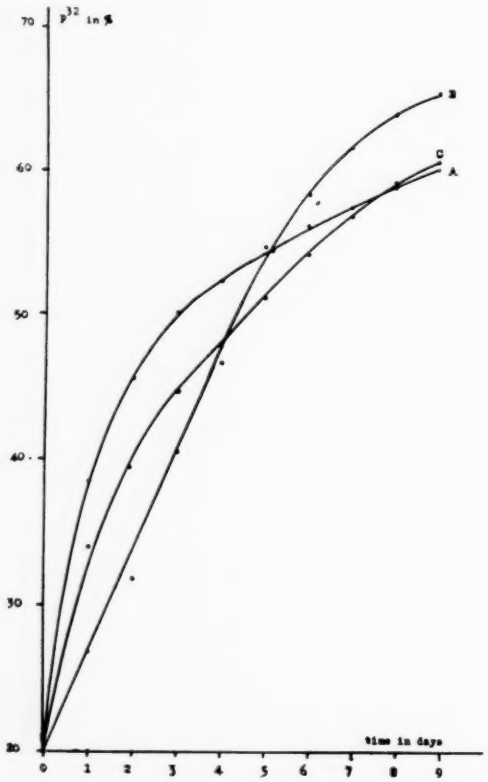


FIG. 5. EXCRETION OF P^{32} IN URINE
The excretion of radioactivity is expressed as percentage of the dose injected.

sirable to repeat in particular the experiments on plasma and red cell turnover using longer periods of observation. For this purpose a higher initial radioactivity of the labelled material was required. This could be achieved by injecting a preparation of a specific activity of approximately 200 μc. per mg. DFP. Two patients of the City Hospital of the Hague were thus treated. One was an 80-year-old man suffering from arteriosclerosis cerebri (D); the other (E) was a 63-year-old woman who, at the time the experiment was started, had completely recovered from an attack of apoplexia and was considered normal. They received dosages of 0.44 and 0.51 mg. of DFP^{32}, respectively.

Resorption of DFP

Blood samples from the fingertip from A and B were examined for radioactivity at 15-minute intervals. Figure 1 shows the course of the radioactivity of the whole blood in counts per minute during an observation period of 130 minutes. There are two main differences between the curves obtained from A and B. Firstly, the maximal activity is reached after 30 minutes in Case A and only after 90 minutes in Case B. Secondly, the level of radioactivity is much higher in the latter case in spite of the fact that only one-eighth of the amount of radioactivity had been injected. These differences are readily explained as the result of the pretreatment with OMPA to which A had been subjected. This treatment has certainly resulted in blocking of the majority of the sites present on proteins of blood plasma and cells which are capable of reacting with DFP. These sites are readily accessible in Case B where they react with DFP to form the irreversible protein-DFP-reaction products well known from cholinesterase inhibition studies. The unbound DFP is assumed to be rapidly removed by the liver, the kidney, and other organs and the difference in level of curves A and B is thus explained on account of differences in bound DFP content of the blood. If the supposition is right that the protein-DFP linkages are irreversible, radioactivity will disappear at the rate of replacement of those proteins by newly formed molecules.

Binding of P^{32} to elements of the blood

For this experiment blood samples from A, B, D, and E were drawn at intervals. After separation of red cells and plasma the amount of DFP in micrograms that had reacted with plasma and cells, respectively, was calculated from the observed radioactivity.

Results are expressed in Figures 2 and 3. It is apparent that, in spite of the larger amount of DFP injected into A, the plasma and red cells harbour considerably less radioactivity than those of B, D, and E, as would be expected from the results presented in the previous passage.

Red cell and plasma cholinesterase activity was measured in the blood of B (Figure 4). A marked inhibition of plasma cholinesterase is observed down to approximately 10 per cent of the initial value. Low values are still observed on the eighth day. Unfortunately at that time it was necessary to start neostigmine treatment so that the further

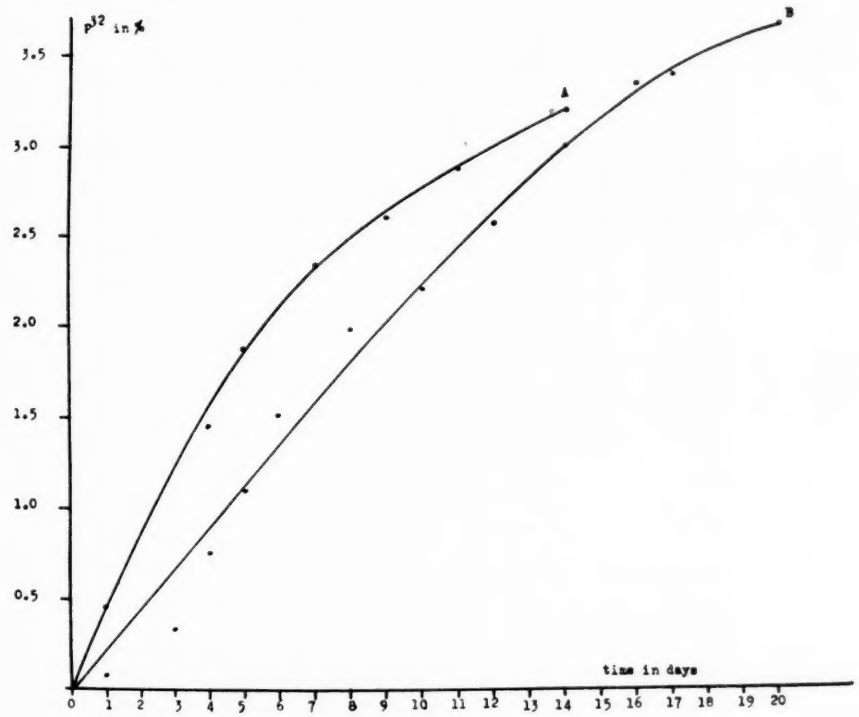


FIG. 6. EXCRETION OF P^{32} IN FECES

The excretion of radioactivity is expressed as percentage of the dose injected.

course of the curve is not fit for detailed analysis. Slow restoration of activity seems however apparent after the twelfth day. The curve for the red cell cholinesterase, which is known to be less sensitive to DFP than the plasma enzyme, shows only a shallow and transitory dip. Moreover it occurred at least 24 hours after the injection. It seems highly unlikely that this transitory dip, occurring after a latent period on a dose of DFP of only 240 micrograms should be attributed to a true DFP inhibition of red cell cholinesterase.

Excretion of DFP^{32}

Radioactivity was measured in the urine of A, B, and C and also in the feces of A and B. In all cases, 60 to 70 per cent of the P^{32} injected as DFP appeared in the urine during the first 10 days (Figure 5). One-third was found in the urine in the first 24 hours. The amount of P^{32} excreted in the urine of A was slightly greater in the first few days and slightly less after the first three to four days compared with B and C. This difference may be due to the OMPA treatment in case A, resulting in a modification of the ratio protein-bound/free P^{32}.

During the observation period less than 5 per cent of the injected radioactivity was recovered in the feces (Figure 6).

Paper chromatography of urine

The results so far reported provide only information on the distribution of the P^{32} incorporated in the injected DFP molecules. They convey nothing concerning the chemical alterations of the DFP molecule between its injection and its excretion. Therefore, the urine was further analysed by paper chromatography to elucidate the chemical character of the radioactive compounds it contained. Portions of urine of B and C collected during the first days after the injection were analysed. Portions of the same urine mixed with diisopropylphosphate (DIP) monoisopropylphos-

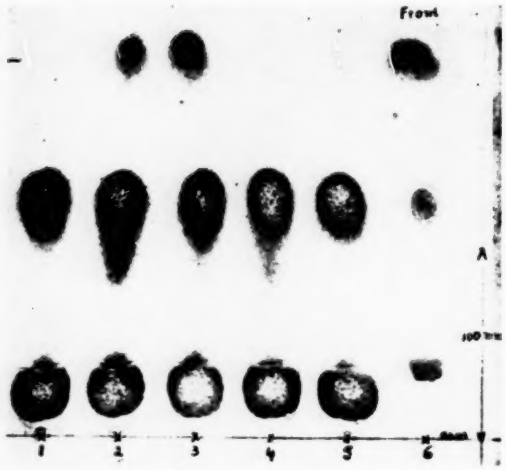


FIG. 7a. PAPER CHROMATOGRAM OF THE URINE OF C

Solvent: butanol-acetic acid-water.

1 urine
2 urine + DIP (diisopropylphosphate) + MIP (monoisopropylphosphate) + AP (anorganic phosphate)
3 urine + DIP

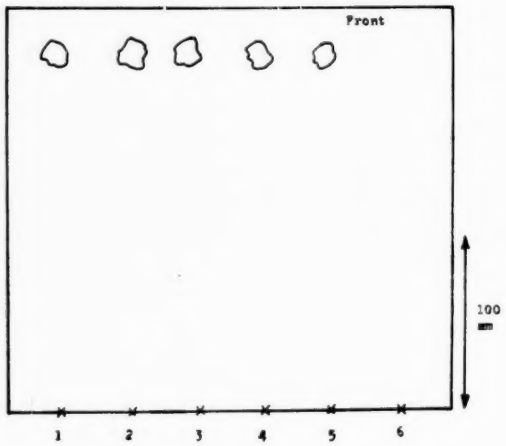


FIG. 7b. RADIOGRAM OF THE CHROMATOGRAM OF FIGURE 7a

4 urine + MIP
5 urine + AP
6 water + DIP + MIP + AP

phate (MIP) and inorganic phosphate were run simultaneously. These substances were considered to be the most likely phosphates to occur in the urine as a result of DFP break-down in the body.

In Figure 7 a chromatogram is presented of five samples of urine of C with additions as indicated. Number 6 is a control in water.

Unmixed urine (No. 1 in Figure 7) shows two large unidentified spots. Comparison with numbers 2, 5, and 6 suggests that the upper part of the lower spot contains probably inorganic phosphate. MIP is located immediately under the upper spot (2, 4, and 6) whereas DIP travels in the front (2, 3, and 6). The original urine (1) cannot be expected to give readily demonstrable spots resulting from DFP or its break-down products because the technique used is not sensitive enough to give a colour with the traces of phosphate^{32} excreted in 40 μl. of urine (see Figure 5).

If, however, an x-ray film is exposed to the same chromatogram, an autoradiogram is obtained. A drawing of this radiogram is presented in Figure 7b. It proves conclusively that the injected DFP appears in the urine in only one form, DIP. It is concluded that at least the main part of the injected DFP is transformed into DIP and excreted as such. It should be noted that these results were obtained on urine of the first days following injection of DFP^{32}. Later, radioactivity becomes too slight to measure dependably. The possibility that in later periods other products appear can, therefore, not be entirely excluded. It could be argued that some of the injected DFP^{32} might be eliminated as such. This product would not appear on the chromatogram because of its volatility. A watery solution of DFP^{32} brought on filter paper rapidly loses its radioactivity due to this volatility. When a drop of fresh urine of C was treated in this way, no radioactivity disappeared on drying, showing that no free DFP was present nor any other volatile P^{32} containing compound.

DISCUSSION

The results obtained on the normal individuals C and E did not differ essentially from those observed in the patients A, B, and D. Such differences as were found between A on the one hand and B, C, D, and E on the other could be easily explained by the OMPA treatment of A prior to the administration of DFP.

The following picture of the fate of DFP may be tentatively suggested as a result of the present

experience with humans and by earlier experiments on animals by other authors (Figure 8). DFP is rapidly absorbed from the intramuscular depot. The presence of radioactive material in the blood can be demonstrated a few minutes after the injection. At the dosage used, maximal radioactivity in the blood is observed after 90 minutes, after which a slow decline occurs, indicating that removal now is predominant over resorption. Free DFP disappears rapidly from the circulation into such organs as kidney, liver, and lung which have been shown to contain radioactivity in similar experiments on rabbits described by Jandorf and McNamarra (3). A large fraction is probably hydrolyzed in the tissues to DIP and excreted into the urine and to a far less extent into the feces. The hydrolysis may be spontaneous but is more likely to be essentially enzymatic. The enzyme system responsible is probably the fluorophosphatase, present in liver and kidney, as described by Mazur (4).

A certain amount of DFP^{32} reacts with the proteins of red cells, blood plasma and tissues. It is implied in the scheme of Figure 8 that this reaction is irreversible and that the reaction products are stable. This assumption is based on many studies reported in the literature, where it has been shown that a number of animal proteins, such as chymotrypsin, trypsin, cholinesterases, aliesterases etc. react with DFP. In all these instances the reaction was irreversible and the reaction product stable.

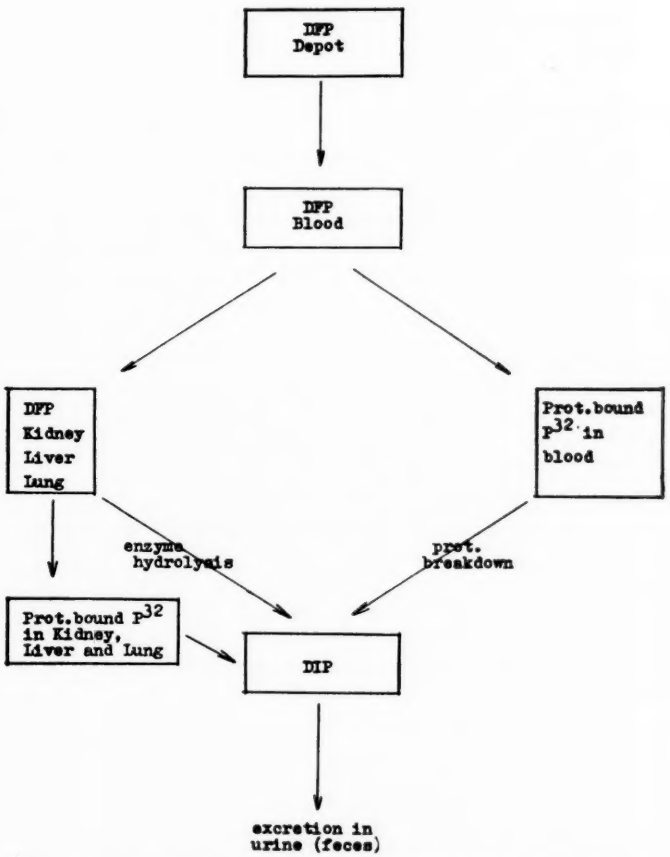
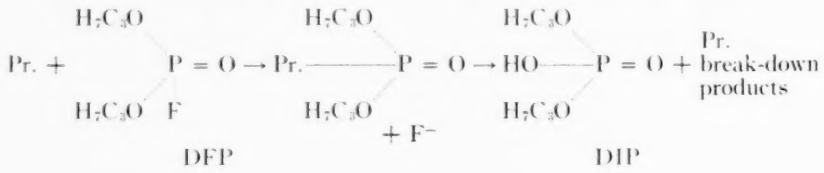


FIG. 8. TENTATIVE DIAGRAM OF THE METABOLIC FATE OF DFP IN THE HUMAN BODY

It seems likely that this protein-DFP interaction bears the same character as the reaction between DFP and chymotrypsin described by Jansen, Nutting, Jang, and Balls (5, 6). It was found that in this reaction DFP lost its fluorine atom whereas the diisopropylphosphate moiety became firmly attached to the protein. As a result of the irreversible character of this linkage further metabolism of the protein bound P^{32} containing moiety is henceforth dependent on the break-down of the protein molecule to which it is attached. When this break-down occurs, again DIP is the product formed.



Pr. = protein

All processes involved result, therefore, in a single final product, the DIP, which is excreted essentially in the urine. DIP, once formed, is not to any extent bound or stored in the body, as has been demonstrated by Jandorf and McNamarra (3). It is not further metabolised and quickly disposed of. It does not give rise to any P^{32} containing compound which is actively involved in body metabolism.

The rapid irreversible reaction of DFP^{32} with certain body proteins suggests its use for the labelling of these proteins. Since, for instance, the radioactivity of labelled red cells can only be reversed by the ultimate destruction of these cells, simple estimations, during a certain period, of red cell linked radioactivity after injection of DFP^{32} should provide information on the rate of break-down of these cells and their life span. In Figure 3 the concentration of red cell linked P^{32} in the subjects D and E is plotted against the time. It will be seen that these two cases have been studied for a period of as long as three months. The P^{32} content of the red cells had reached half its initial value after 58.1 and 64.4 days in D and E, respectively, corresponding to a mean life span of 116 and 129 days. These values compare satisfactorily with those reported in the literature (mean life span 120 days [7]). From the curves of Figure 3 it would seem that during the first 7 to 10 days after the injection of DFP^{32} the red cell bound radioactivity remains constant. After that period radioactivity falls at a steady rate which may be measured and used for calculations of life span. The reason for this initial constancy has not been studied. Similarly irreversible combination of injected DFP^{32} with blood plasma can be used for the calculation of plasma protein turnover (Figure 2). In this case, the curves depicting the elimination of the labelled plasma protein fraction were exponential reflecting random destruction. They were plotted logarithmically to produce straight lines from which the half life time could be calculated. Values of 12 to 14 days were found for the half life of this blood plasma component in the patients A, B, D, and E (13.7, 13.7, 13.1, and 12.5 days, respectively).

It should be kept in mind that the method outlined above can only provide information on the turnover of that plasma component which reacts with DFP. It is probably mainly or exclusively the pseudo-cholinesterase component of human plasma. The half life of 12 to 14 days found by us for this plasma fraction in adult humans is comparable with the figures given by Dixon, Talmage, Maurer, and Deichmiller (8) for gamma globulin in human adults (13 days) and by London (9) for serum proteins (albumin 20 days, β and γ globulin 12 days, γ globulin 19 days). The most important advantage, next to its simplicity, of the present procedure over methods so far described is probably the fact that only one metabolite, the DIP, results from the impact of DFP on the organism, and that this DIP never takes part in any form in further metabolic processes but is rapidly eliminated. Therefore, no active metabolic pool can get polluted with P^{32} and resynthesis of red cells and plasma involving radioactive material is not likely to interfere with the turnover estimations. It might be advantageous to tag plasma or red cells *in vitro* by DFP^{32} and then inject the reaction

products. This possibility has not been explored but seems worth considering. This procedure might also be useful for the determination of red cell and plasma volume, particularly because of the irreversibility of the P^{32} label.

The possibility has not been ruled out as yet that the reaction-products of DFP and plasma or red cells do not behave entirely like the unlabelled structures. More evidence is required before the method can be confidently recommended as a reliable general technique. The values obtained seem reasonable compared with those described in the literature and the regular pattern of the curves provide some confidence that the methods might be useful.

With the dosages used no symptoms of toxicity have been observed.

SUMMARY

1. Five human subjects were injected intramuscularly with varying non-toxic doses of DFP^{32} (diisopropylfluorophosphonate labelled with P^{32}).

2. Under normal conditions radioactivity originating from DFP could be demonstrated in the peripheral blood within a few minutes; maximum radioactivity was reached in 90 minutes.

3. A small portion of the injected P^{32} was irreversibly bound to red cells and plasma. This fraction disappeared from the blood at a rate corresponding to the normal replacement of these elements.

4. This relationship could be exploited to calculate the rate of replacement of a plasma protein and the mean life span of red cells.

Values of 12 to 14 days were found for the half life of this plasma component in four persons.

A mean red cell life span of 116 and 129 days, respectively, was found in two subjects.

5. Injected DFP is excreted in the urine as diisopropylphosphate (60 to 70 per cent in the first ten days); less than 5 per cent of the P^{32} injected as DFP is recovered in the feces during that period.

6. In one person DFP receptors were blocked by treatment with OMPA (octamethylpyrophosphoramide) prior to the injection of DFP^{32}. In this case less radioactivity was bound to plasma and red cells and maximal radioactivity in the blood was reached as soon as 30 minutes after the injection of DFP. Excretion was slightly accelerated. These data are understandable on account of the relative decrease of firmly bound and increase of free DFP^{32} as a result of the OMPA pretreatment. The free DFP is more readily removed from the blood than the bound fraction.

7. It is suggested that labelling by means of DFP^{32} may also be used for the evaluation of red cell and plasma volume.

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Issued February, 1954

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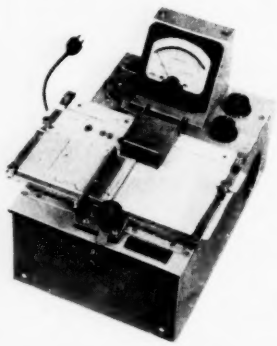

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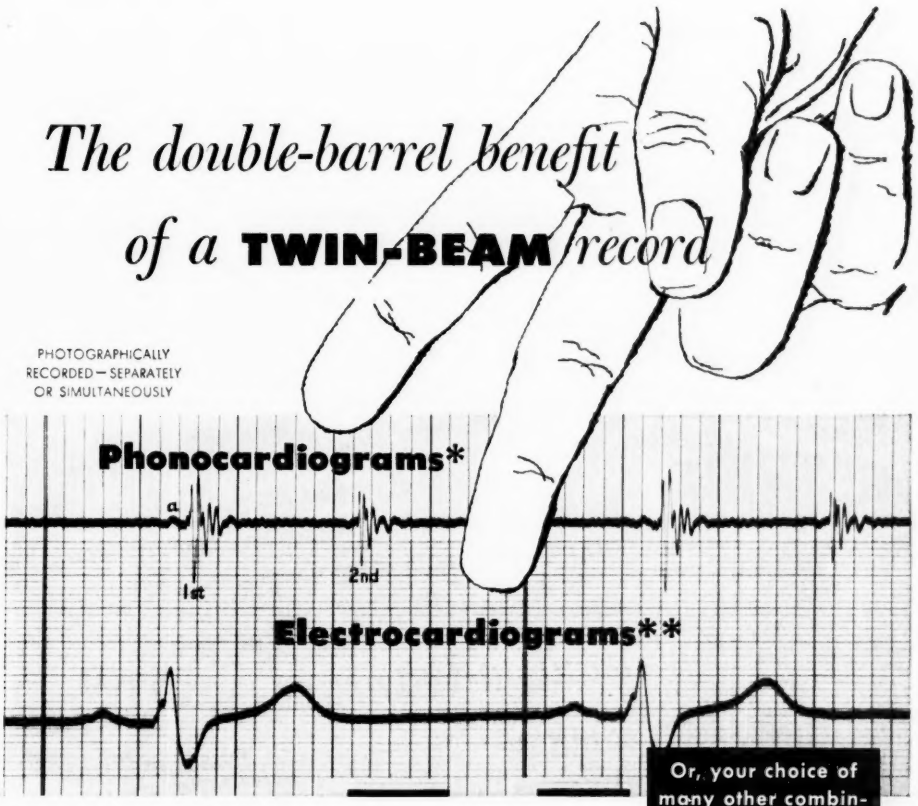

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are fully detailed recordings of ALL of the heart sounds and murmurs present. The location, pitch, duration, and intensity of a murmur are reproduced with the same completeness of detail as are the auricular, first and second sounds of the normal record shown above.

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